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International Society for Stem Cell Research

June 13 – 16, 2012

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Poster Session Abstracts - Volume 1

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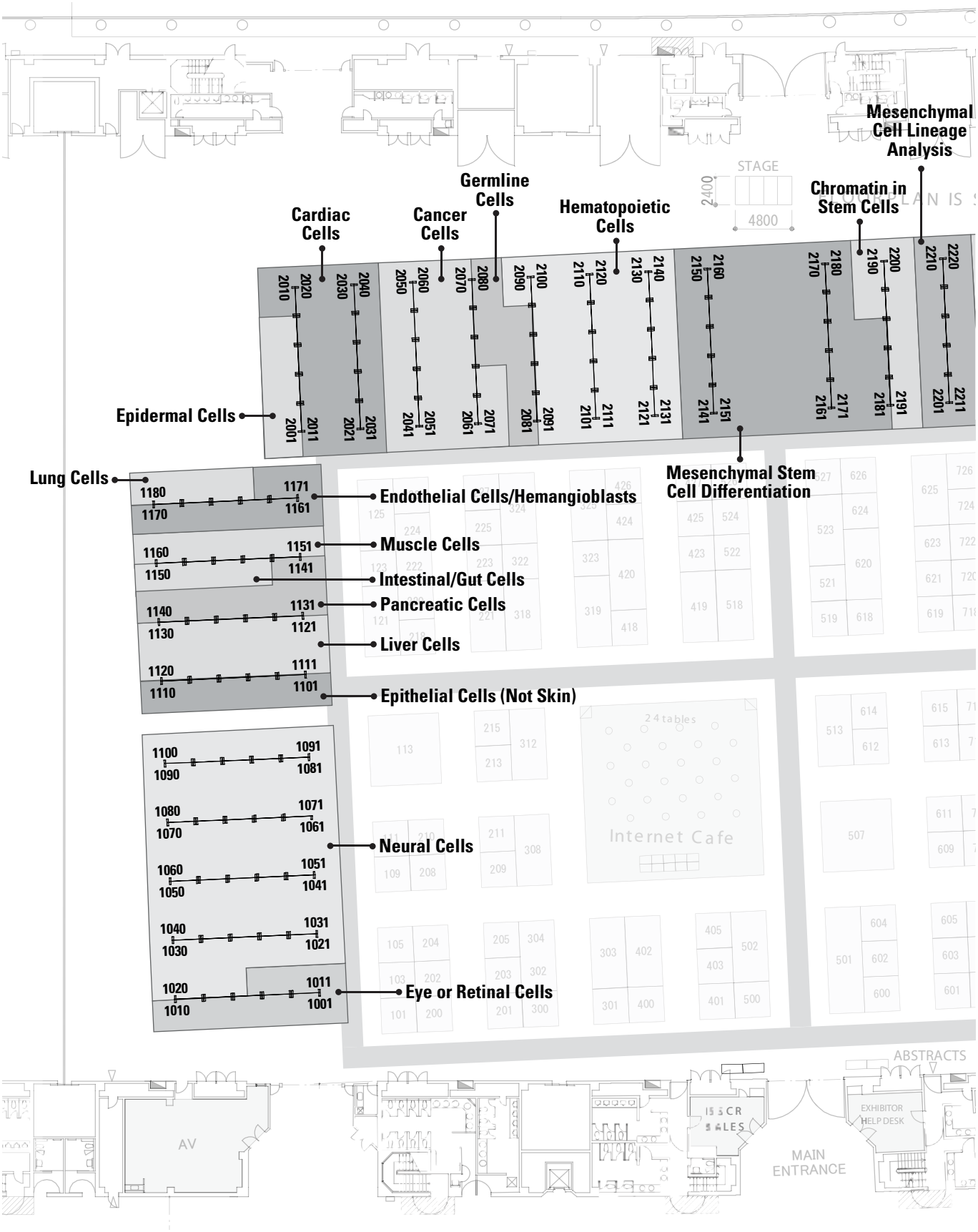

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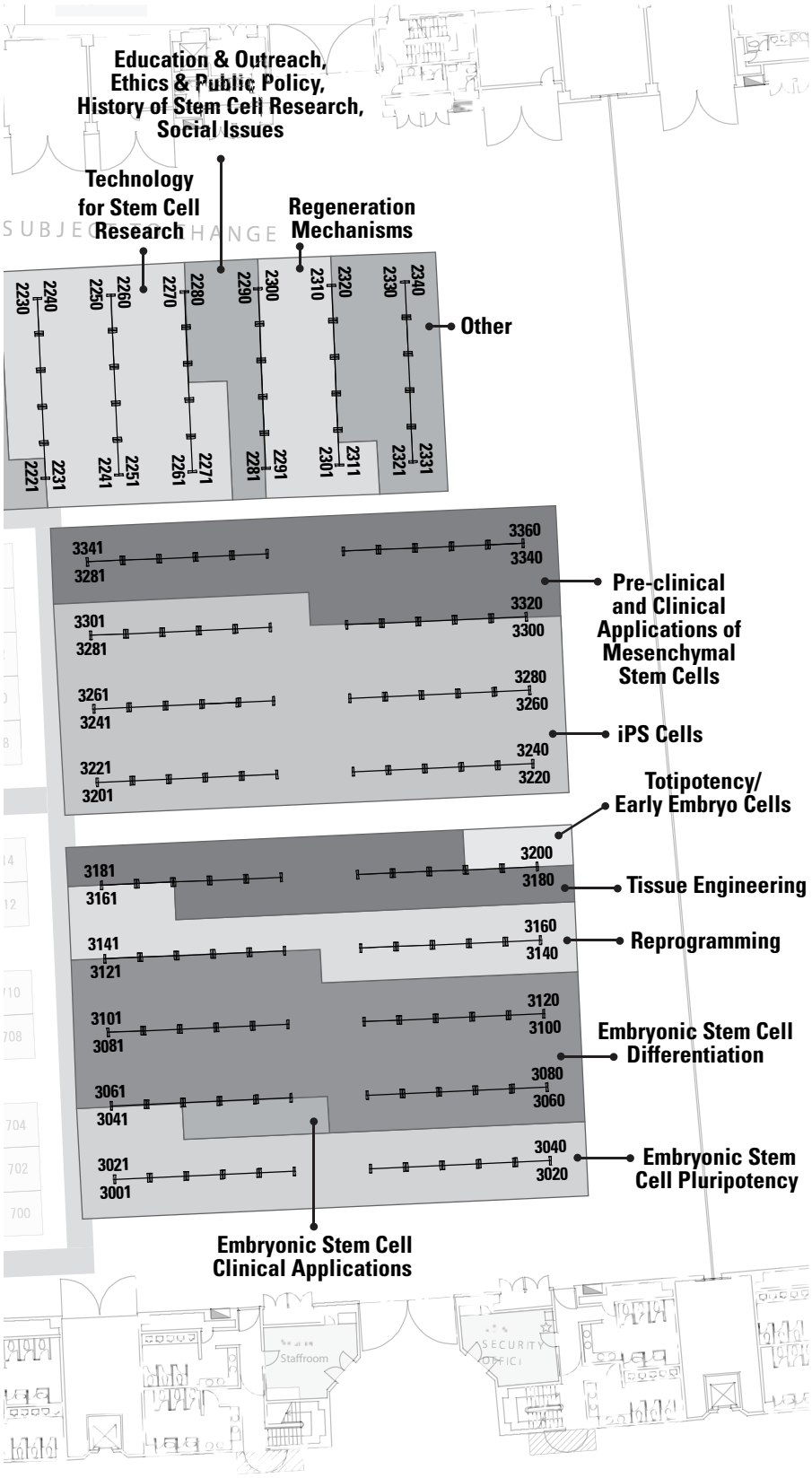

Poster Boards by Topic

Posters 1001 – 1180

Posters 2001 – 2340

Posters 3001 – 3360

NOTE: The presenters of the **ODD-numbered** posters are asked to present for the first hour of the receptions. The presenters of the **EVEN-numbered** posters are asked to present for the second hour of the receptions.

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Detailed Program and Abstracts — Thursday, June 14

Eye or Retinal Cells

Poster Board Number: T-1001

CELL SURFACE MARKERS OF HUMAN RETINAL PROGENITOR CELLS

Baranov, Petr[1], Melo, Gustavo[1], Stevanato, Lara[2], Patel, Sara[2], Sinden, John[2], Young, Michael[1]

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Purpose: Retinal degenerative disorders remain the leading cause for vision loss in developed countries. Since the regenerative capacity of human neural retina is highly limited, one viable treatment option is cellular replacement. Retinal progenitor cells (RPC), isolated from the fetal retina, have been shown to form new functioning photoreceptors and restore retinal function following transplantation into retinal degenerative hosts. However, compared to mouse RPC, not much is known about the hRPC phenotype. The aim of this study was to comprehensively describe the phenotype of GMP-manufactured hRPCs as a drug product for retinal degenerative disorders. Methods: hRPC were isolated from human neural retina at 18 week of gestational age and expanded under low-oxygen (3%) conditions up to passage 9. Karyotype analysis, immunocytochemistry and flow cytometry analysis for stemness, eye field, neuronal, proliferation and retinal cell markers were performed. Also, functional properties were investigated using calcium imaging and differentiation assay. Results: hRPC at passage 9 were found to have no chromosome abnormalities, and responded to L-glutamate, L-citrate and NMDA-Glycine. Immunostaining found the presence of Sox2, Klf4, Recoverin, Otx2, Pax6, Ki67, PCNA, CyclinD1, b3 tubulin, NF200, Nestin, Vimentin, SSEA4, CD24, Crx, Nrl, CD73, PSA-NCAM, PTK7. We were unable to detect significant expression of mature photoreceptor markers such as Rhodopsin, Opsin Red/Green, Nr2e3, or Rod Outer Membrane. Also, we found hRPC to be negative for markers specific for neural stem cells (CD133, CD15), retinal pigment epithelia (RPE65), and glial progenitors (A2B5, CD38). However, upon differentiation *in vitro* we observed more than 50% of cells to express mature photoreceptor markers (Rhodopsin, Opsin Blue, Opsin Red/Green).Conclusions: The phenotype of hRPC may be used to compare different cell products to be prepared for transplantation. The presence of CD24 and CD73, found on mouse photoreceptor precursors, suggest the potential of this population to form rods and cones and PSA-NCAM expression is characteristic of cells able to integrate into host retina.

Poster Board Number: T-1002

HUMAN PLURIPOTENT STEM CELL DERIVED RETINAL PIGMENT EPITHELIUM CULTURE ON TRANSPLANTABLE BIOMEMRANE

Hiidenmaa, Hanna[1], Subrizi, Astrid[2], Ilmarinen, Tanja[1], Yliperttula, Marjo[3], Uusitalo, Hannu[4], Urtti, Arto[2], Skottman, Heli[1]

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Human embryonic stem cells (hESCs) are differentiated toward retinal pigment epithelium (RPE) in several laboratories aiming to treat degenerative diseases of the retina, and create an *in vitro* model of the human retina. Injected hESC-RPE cells have been shown to improve visual functions. However, in most of the studies hESC-RPE cell integration has been unsatisfactory and the cells have vanished in a few months after the injections. One reason for poor RPE survival could be aged or diseased basal lamina, the Bruch´s membrane. Here we report successful hESC-RPE growth on transplantable biomembrane, which could be used to enhance RPE integration and survival in the host tissue. We studied the growth and maturation of hESC-RPE on porous biomembrane fabricated from clinically accepted polyimide (PI). In addition, we studied the role of several extracellular matrix proteins and protein mixtures in supporting hESC-RPE attachment, growth, and maturation. Spontaneously differentiated pigmented cells were seeded on PI membranes with different coatings. The cell growth and pigmentation was analyzed weekly under microscope. After eight to twelve weeks of culture, the expression of RPE specific genes and proteins was analyzed. The tightness of the epithelium was evaluated by measuring transepithelial electrical resistance (TEER) and cell permeability of 6-carboxyfluorescein. Co-culture with rat retina explants was used to test the functionality of hESC-RPE. PI biomembrane alone without coating did not support hESC-RPE attachment or growth. Nonetheless, over half of the tested coatings improved cell attachment and RPE maturation. hESC-RPE cells were positive for common characteristics of native RPE cells, such as cuboidal morphology, pigmentation, gene and protein expression, and polarization. hESC-RPE cultured on coated PI membranes formed tight epithelium based on protein expression, TEER and permeability values. In addition, hESC-RPE showed functionality by phagocytosing photopigments of the rat retina. This study shows that ECM component coated PI membrane supports hESC-RPE culture and maturation and would be a potential scaffold for further hESC-RPE transplantation studies.

Poster Board Number: T-1003

DOWNREGULATION OF CANONICAL WNT SIGNALLING IN ADULT HUMAN MÜLLER STEM CELLS LEADS TO EXPRESSION OF RGC MARKERS *IN VITRO*

Becker, Silke, Guo, Christina, Wong, Aaron J., Eastlake, Karen, Jayaram, Hari, Limb, G Astrid

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Background: Müller glia cells with stem cell characteristics have been shown to differentiate into various types of retinal neurons in the lower vertebrate retina. Although these cells have been identified in the adult human retina, they are unable to repair retinal damage in situ. However, human Müller stem cells (MSC) have been shown to differentiate towards retinal neural phenotypes including retinal ganglion cells and photoreceptor precursors *in vitro*. Regulation of the Wnt signalling pathway has previously been shown to be critical for maintaining retinal stem cells as well as facilitating their proliferation and differentiation into retinal neurons during embryonic development. The role of Wnt signalling in human MSC for the maintenance of a stem cell phenotype and for their differentiation into retinal neurons is currently unknown. The present study aimed to determine the function of the canonical Wnt signalling pathway in human MSC and the effects of Wnt inhibition on the development of differentiation protocols towards retinal neural phenotypes. Methods: Expression of genes and proteins of the canonical Wnt signalling pathway was examined using RT-PCR, western blotting and immunohistochemistry. Downregulation of Wnt proteins was performed in cells cultured for 7 days in the presence of Dickkopf-1 (Dkk-1), an inhibitor of the canonical Wnt pathway. For RT-PCR experiments total RNA was extracted, reverse transcribed and PCR was performed. PCR products were analysed by gel electrophoresis and quantified by densitometry. For western blotting experiments total protein was extracted, run on SDS-PAGE and protein expression was detected with specific

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primary antibodies. Cells treated or untreated with Dkk-1 were examined for their expression of markers of retinal ganglion cells, Wnt 2B and β-catenin using immunohistochemistry and confocal microscopy.Results: Various components of the Wnt pathway, such as Wnt5B, FZD1, FZD4, FZD7, β-catenin and LEF1 were shown to be expressed by MSC. Conversely mRNA for Wnt3a and FZD5 was not detected. High levels of β-catenin were localized in the nuclei and cytoplasm of most cells examined. Downregulation of the canonical Wnt pathway using Dkk-1, greatly attenuated the expression of β-catenin and Wnt2B. Treatment with Dkk-1 also resulted in a marked reduction in proliferating cells as determined by positive staining for the proliferative marker Ki-67. Importantly, the expression of proteins typically expressed in retinal ganglion cells was augmented in MSC treated with Dkk-1, as shown by RT-PCR and western blotting. In particular, the ganglion cell markers Isl-1, HUD and Brn3b were significantly increased in MSC following inhibition of the Wnt pathway. Conclusions: The study showed that various components of the Wnt pathway are expressed in human MSC and suggests that constitutive activity of the canonical Wnt pathway may promote the proliferation of undifferentiated Müller stem cells. Conversely, inhibition of the canonical Wnt pathway attenuated cell proliferation and induced differentiation of Müller glia cells towards retinal neuronal phenotype as judged by their increased expression of retinal ganglion cell markers. Inhibition of the canonical Wnt pathway in human Müller stem cells may prove to be an important tool for the development of cell populations enriched in retinal neurons and may be valuable for the development of retinal cell transplantation strategies in the treatment of retinal degenerative diseases.

Poster Board Number: T-1004

NEUROPROTECTIVE EFFECTS OF HUMAN NEURONAL PROGENITOR CELLS EXPRESSING IGF-1 AND IGFBPL-1 ON MODELS OF RETINAL GANGLION CELL LOSS

Ma, Jie[1], Guo, Chenying[1], Chen, Dong Feng[1], Chen, Guochun[2], Cyr, Desirée[1], Lashkari, Kameran[1]

[1]Schepens Eye Research Institute, Boston, MA, USA, [2]The Second Xiangya Hospital, Central South University, Changsha, China

We have recently reported that neural progenitor cells isolate from human persistent fetal vasculature (NPPFV) can differentiate into retinal neurons and specifically, retinal ganglion-like cells (RGC) after transplantation into the mouse vitreous.1 Immunostaining indicated that the NPPFV cells penetrated the inner retina and integrated with the host inner nerve (plexiform) fiber layer. NPPFVs were also transplanted into a mouse model of pigmentary glaucoma (DBA/2J), in which there is progressive and complete loss of host RGCs. Interestingly, host RGCs that were in apposition to NPPFVs survived this harsh ocular hypertensive environment while other RGCs underwent apoptosis. Based on these and other *in vitro* observations we hypothesized that NPPFVs confer a relative degree of neuroprotection to host RGCs through secretion of a known neuroprotective factor, insulin-like growth factor-1 (IGF-1) and its binding protein, IGFBPL-1. It has been shown that both factors can significantly enhance axon growth of RGCs and enhance their survival *in vitro*. To enhance the neuroprotective effects of NPPFVs we questioned whether increased production and secretion of IGF-1 and IGFBPL-1 could confer more global neuroprotection against stress-induced RGC loss. IGF-1 and IGFBPL-1 cDNAs were cloned into a plasmid carrying a fluorescence reporter gene to generate fluorescent fusion proteins. Plasmid encoding IGF-1 or IGFBPL-1 gene was constructed and spliced into pJ603-neo vectors expressing a red (RFP, TD-tomato) or green (GFP) fluorescent protein, respectively. Cells were transfected by electroporation using one or both vectors and examined under fluorescence microscopy. Transfected cells were enriched by antibiotic selection and subjected to RT-qPCR and ELISA for quantitation of IGF-1 and IGFBPL-1 expression. Transfected cells, NPPFVIGF-1, NPPFVIGFBLP-1 and NPPFVIGF-1/GFBLP-1 were studied under a co-culture condition with RGCs and evaluated for neuronal morphology, axonal growth and apoptosis. Double transfection of these genes yielded significantly increase production of IGF-1 and IGFBPL-1 (15.8 fold vs. 3.7 for naïve NPPFVs in combination). ELISA also indicated that transfected NPPFVs secreted both gene products in culture. In co-culture condition, transfected NPPFV cells improved morphology of resident RGCs and decreased apoptotic signals. Our data indicate that neuroprotective genes such as IGF-1 and its binding protein, IGFBPL-1 could be successfully transfected and expressed into neuronal progenitor cells such as NPPFVs. Expression of these proteins improves neuronal morphology, axonal growth and survival of RGCs. Overall, NPPFVs expressing neuroprotective transgenes may be good candidate cells for transplantation of the inner retinal to confer increased neuroprotection to host RGCs under disease conditions. 1 Cell Transplantation, in press, 2012. Corresponding author: Lashkari, Kameran

Poster Board Number: T-1005

HISTONE MARKS PREDICT CELL PLASTICITY OF THE ADULT HUMAN RETINAL PIGMENT EPITHELIUM

Blenkinsop, Timothy A.[1], Rada Iglesias, Alvaro[2], Charniga, Carol[1], Wysocka, Joanna[2], Temple, Sally[1]

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Retinal pigment epithelial (RPE) cells are one of the few cell types well recognized to change fate in lower vertebrates. We recently showed that adult human RPE contains a minor sub-population of cells that can be activated into self-renewing multipotent stem cells. These RPESCs can be derived from donors as old as 99 years, and can differentiate down RPE, neural or mesenchymal lineages using appropriate differentiation media. Addressing whether the observation of mesenchymal fates was due to the multipotency of the RPE cells or to a contaminating cell type, for example MSCs, in the cultures was critical. Therefore, we demonstrated that single, cloned RPE cells exhibit this ability, irrefutably demonstrating that they are multipotent cells. We therefore sought to understand the basis for this observed plasticity. Histone marks at promoter and enhancer sites can reveal both genes that are actively being transcribed - i.e. active, and genes that are poised - i.e. currently inactive, but can be activated by under appropriate conditions. Recently, the histone H3K27me3 and H3K4me3 marks has been shown to be concomitantly enriched in so called bivalent promoter regions, which in human embryonic stem cells (hESCs) are thought to poise developmental genes for subsequent activation. Similarly, distal enhancers in hESC are enriched in either H3K27ac or H3K27me3, depending if such regulatory elements control transcriptionally active or poised genes, respectively. Therefore, we hypothesized that the combination of histone marks at promoter and enhancer regions could predict poised genes in RPE that can become active in response to stimuli and that could explain RPE plasticity. We cultured human retinal pigment epithelium into polarized layers and verified their terminal differentiation throuh transepithelial resistance (TER) in Ω.cm2. These monolayers expressed TER of > 200Ω.cm2 similar to native RPE. We also confirmed their identity immunohistochemically, by quantitative PCR, and using a phagocytosis assay. We then conducted H3K27ac, H3K27me3, H3K4me3 and

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H3K4me1 chromatin immunoprecipitation-sequencing (ChIP-seq) on adult human RPE monolayers. We found that RPE specific genes possess H3K27ac and H3K4me3 marks at their promoter regions, whereas the genes we have found to increase expression during differentiation of RPE, for example RUNX2 during osteogenic differentiation, display a bivalent promoter enriched in both H3K27me3 and H3K4me3. In addition, we identified a large number of putative enhancer elements, whose relevance and specificity are currently being evaluated. These results suggest that poised promoter and enhancer signatures may facilitate understanding of the molecular basis of RPESC plasticity.

Poster Board Number: T-1006

RETINAL TRANSPLANTATION OF PHOTORECEPTOR PRECURSORS DERIVED FROM MOUSE EMBRYONIC STEM CELLS UNDER HYPOXIC CONDITIONS IMPROVED THE EFFICIENCY OF DIFFERENTIATION

Garita-Hernandez, Marcela[1], Díaz-Corrales, Francisco[1], Lukovic, Dunja[1], Gonzalez-Guede, Irene[1], Massalini, Simone[1], Díez-Llobet, Andrea[1], Erceg, Slaven[2], Bhattacharya, Shom S.[2]

[1]Cell Therapy and Regenerative Medicine, Cabimer, Seville, Spain, [2]Cell Therapy and Regenerative Medicine, Cabimer, Medical Genome Project, Seville, Spain

Retinal dystrophies such as Retinitis pigmentosa (RP) and age-related macular degeneration (AMD) are among the leading causes of permanent blindness and are characterized by the progressive dysfunction and death of the light sensing photoreceptor cells of the retina. Due to the limited intrinsic capacity of the mammalian retina to regenerate, the use of embryonic stem cells (ESC) as an unlimited source to replenish the lost cells has represented a main objective for the scientific community. Despite great advances in the field of differentiation of ESC towards photoreceptors in the recent years, few drawbacks remain unresolved. Such as, efficiency, purity of the population and the constant worry that once differentiated, cells would not be able to integrate into the host retina. We have designed a new protocol to differentiate mouse ESC (mESC), involving the control of the microenvironment, particularly the oxygen tension of the retinal niche-specific conditions, as well as the manipulation of key signaling pathways involved during normal retinal development. We have observed that hypoxia not only increases the efficiency of differentiation towards photoreceptors, in terms of the number of Rhodopsin/Recoverin and Opsin-S positive cells but it also improves the modeling of retinogenesis by decreasing the time necessary to acquire each specific retinal phenotype *in vitro*. Specific transcription factors associated with each stage of retinal differentiation such as eye field (Rax, Six3), optic cup (Pax6, Mitf, Chx10), photoreceptor precursors (Nrl, Crx) and even mature photoreceptors (Rhodopsin, Recoverin and Opsin-S) were upregulated earlier and their levels of expression were significantly higher than those reached under normoxic conditions as determined by qPCR. Immunofluorescence analysis also confirmed a significant higher yield of positive cells for Rax, Crx, Opsin-S, and Rhodopsin/Recoverin positive cells derived under hypoxia. Furthermore, differentiation of mESC cells following our protocol under hypoxia retained a higher proliferative capacity *in vitro* and when photoreceptor precursors were transplanted in the subretinal space of wild type mice they differentiated mainly towards Recoverin/Rhodopsin positive cells, migrated and integrated into the host retina and in some cases acquired the morphology of mature rods with formation of structures suggesting outer segments. We believe our results support the hypothesis that hypoxia is necessary to induce a higher efficiency of differentiation of embryonic stem cells towards retinal phenotypes. Purification of these specific retinal progenitors will allow us to define conditions to expand a homogenous population that could be further differentiated into fully mature photoreceptors

Poster Board Number: T-1007

EXPLORING THE USE OF HUMAN VERY SMALL EMBRYONIC-LIKE STEM CELLS (VSELS) ISOLATED FROM ADULT PERIPHERAL BLOOD FOR THERAPY OF DRY AGE-RELATED MACULAR DEGENERATION (AMD)

Eminli, Sarah[1], Jiang, Caihui[2], Chen, Guochun[2], Baranov, Petr[2], Cyr, Desiree[2], Leary, Elizabeth[1], Yavanian, Gregory[1], Hall, Sean[1], Jiang, Yajuan[1], Marasco, Wayne A.[3], O'Neill, David W.[1], Lashkari, Kameran[2], Young, Michael J.[2]

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Age-related macular degeneration (AMD) is one of the leading causes of incurable blindness in the western world. More than 7 million people in the United States suffer from AMD, and over 2 million over age 50 have progressed to the late stage of the disease, with drastic vision impairment or permanent loss of vision. Atrophic (dry) AMD, the most prevalent form, accounts for 90% of all cases, and is characterized by the progressive deposition of debris underneath the retinal pigment epithelium (RPE) layer, a highly specialized tissue that supports, protects and nurtures the light sensitive photoreceptors of the outer neural retina, leading to their degeneration. End stage AMD occurs when the RPE completely degenerates, with associated loss of photoreceptors and the underlying capillary layer called choriocapillaris. Cell transplantation (RPE, photoreceptor precursors or cells with neuroprotective abilities) could be used to restore sight in advanced AMD by replenishing the subretinal anatomy and re-establishing the functional relationship between RPE and photoreceptors. Recently, Very Small Embryonic-like Stem cells (VSELs) have been identified in human bone marrow and in adult peripheral blood. Human VSELs are populations of small (5 to 9 μm) Lin-, CD45- cells that can express CD133, CD34, CXCR4, as well as high levels of markers characteristic of embryonic stem cells such as Oct-4 and Nanog. To explore the regenerative potential of VSELs in the retina, we transplanted PKH26-labeled enriched human VSELs into the mouse eye -- both by injecting the cells into the vitreous space of immunodeficient mice, and by injecting the cells subretinally in a SCID immunodeficient mouse model of retinal detachment. We assessed the ability of human VSELs to engraft, survive and differentiate into retinal or neuroectodermal cells in the mouse retina. At both 2 and 4 weeks after transplantation the subretinally and intravitreally injected human VSELs were able to engraft, survive and migrate within the retina. Furthermore, subsequent immunohistochemistry analysis revealed that the subretinally transplanted cells were able to differentiate and express markers of retinal stem and developing progenitor cells such as Nestin and PAX6, markers of neuro-ectodermal cells such as MAP2 and beta-3-tubulin, and the early photoreceptor marker, recoverin. These studies indicate that human VSELs can engraft, migrate and differentiate along the retinal lineage. These observations warrant further investigation to evaluate the potential role of these cells to treat AMD.

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Poster Board Number: T-1008

FUNCTIONAL TISSUE ENGINEERED CORNEAL ENDOTHELIUM DERIVED FROM MOUSE CORNEAL STROMA STEM CELLS

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Purpose: Corneal endothelial dysfunction remains a common indication for keratoplasty, accounting for half of the total number of such procedures. Corneal endothelium as well as corneal stroma originates from the neural crest. We previously reported the isolation of Cornea-derived Precursors (COPs), which have characteristics of multipotent neural crest-derived stem cells, from mouse corneal stroma. In this presentation, we report tissue engineered corneal endothelium (TECE) derived from COPs. Methods: We cultured COPs from mouse corneal stroma in the specific endothelium-inducing medium including retinoic acid, glycogen synthase kinase 3-beta inhibitor, and TGF beta-2 for one week to induce TECE. Quantitative RT-PCR was performed to detect markers characterizing corneal endothelium (Na,K-ATPase α1-subunit, carbonic anhydrase, Na,HCO3 co-transporter, collagen IV, collagen VIII, and Pitx2). The pump function attributable to Na,K-ATPase activity of TECE was measured with an Ussing chamber, and compared with that of primary cultured mouse corneal endothelial cells (mCE) and 3T3 cells. Transplantation of TECE sheets into rabbit cornea was performed to evaluate *in vivo* TECE pump function. Results: After one week culture, hexagonal mosaic pattern monolayer TECE was obtained from COPs. Quantitative RT-PCR revealed significant increases of the expressions of all of the above markers. Na,K-ATPase pump activity of TECE (198.4μA/cm2) was significantly higher than COPs (15.2μA/cm2) and 3T3 (40.8μA/cm2) cells, and 1.7-fold higher than cultured mouse corneal endothelial cells (116.3μA/cm2). TECE transplanted into rabbit corneas maintained transparency and corneal thickness (497.5±104.9μm), whereas control corneas without TECE showed marked edema and increased corneal thickness (1105.8±165.9μm). Conclusions: We successfully derived TECE from mouse corneal stromal cells (COPs), which has equal pump function with mCE in vitro and *in vivo*.

Poster Board Number: T-1009

CD44 MEDIATES THE POST-TRANSPLANT SURVIVAL OF ADULT MOUSE RETINAL STEM CELL-DERIVED ROD PHOTORECEPTORS

Ballios, Brian G., Clarke, Laura, Shoichet, Molly S., van der Kooy, Derek

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Adult retinal stem cells (RSCs) derived from the ciliary epithelium of mice can give rise to all retinal cell types. RSC-derived photoreceptors have demonstrated functional recovery in mouse models of disease. The potential of RSC-derived rods in adult mouse transplantation models has been limited by poor cell distribution, survival and integration into host tissue. An injectable and biodegradable hydrogel blend of hyaluronan and methylcellulose (HAMC) has shown promise in overcoming the cell distribution barrier. Here we report a mechanism through which HAMC directly supports the survival and integration of post-mitotic RSC-derived rods *in vitro* and *in vivo*. RSC-derived rods were pre-differentiated on laminin substrate in the presence of taurine/retinoic acid, which increases the percentage of rods differentiating in clonal mouse and human RSC colonies to over 95% of the population. Survival was assessed with ethidium homodimer and phenotype by immunocytochemical (ICC) staining for rhodopsin, Pax6 and RPE65. RSC-derived rods were harvested at various pre-differentiation time points and injected in a vehicle of either HAMC or saline (control) subretinally into adult mice. Cell distribution in host tissue was analyzed 3 weeks post-transplantation among neural retina, subretinal space, and retinal pigment epithelium (RPE) in the HAMC vs. saline vehicles, with or without addition of the glial-specific toxin DL-alpha-aminoadipic acid (AAA). Highest levels of cell survival/integration in neural retina were observed with the transplantation of early post-mitotic RSC-derived rods in HAMC+AAA. Post-mitotic RSC-derived rod photoreceptors showed significantly improved *in vitro* survival in HAMC compared to media alone. ICC and Q-PCR analysis revealed the expression of the hyaluronan receptor CD44 on RSC-derived rods. The binding of HA to CD44 on other cell types is known to influence cellular migration and survival. CD44-/- RSC-derived rods did not exhibit the pro-survival effect seen with HA-containing mixtures *in vitro*, demonstrating the specificity of this interaction. The enhanced survival and neural retinal integration of wild-type RSC-derived rods after transplantation in HAMC+AAA is lost with transplantation of CD44-/- RSC-derived rods. Our results suggest that HAMC directly promotes RSC-derived rod survival *in vitro* and *in vivo* through the CD44 receptor.

Poster Board Number: T-1010

CANONICAL WNT SIGNALING DETERMINES TUMORIGENICITY AND FUNCTION OF TRANSPLANTED ESC-DERIVED RETINAL PROGENITORS

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Tumor formation constitutes a major obstacle to the clinical application of embryonic stem cells (ESCs). Here, we report that further differentiation of ESC-derived neural progenitors into retinal progenitor cells (ESC-RPCs) completely eliminates teratomas in ocular transplantation. However, tumor-like neural overgrowth occurs in 61% of transplanted eyes. Inhibition of Wnt signaling by DKK1 promotes the commitment of ESC-RPCs to more mature retinal cells and reduces the occurrence of tumor-like neural overgrowth to 3%. DKK1-treated ESC-RPCs efficiently integrate to the host retina, form synaptic connections and restore visual function. Mechanistically, canonical Wnt signaling effector Tcf1 directly activates Sox2 and Nestin expression. Silencing Sox2 or Nestin produces phenotypes resembling to those of DKK1 treatment. Furthermore, Tcf1, Sox2 and Nestin are highly expressed in the early retinal development and retinoblastoma. Collectively, this study demonstrates for the first time the pivotal role of Wnt signaling and the Tcf1/β-catenin-Sox2-Nestin cascade in controlling tumorigenicity and the therapeutic effect of transplanted ESC-derived progenitors.

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Poster Board Number: T-1011

PHOTORECEPTORS REINITIATE THE CELL CYCLE MACHINERY DURING DEGENERATION

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Recent data concerning post-mitotic neurons show the reactivation of cell cycle proteins during the process of cell death in several neurodegenerative diseases. We asked whether the control of this cell cycle protein expression may share some common features with the regulation of neural stem cell proliferation. We investigated the retina of Rd1 mice, a model of retinitis pigmentosa, with rod function defects, widely used to study photoreceptor loss. We used different knockout mouse models to reveal whether proteins involved in the cell cycle regulation are responsible for photoreceptor loss in the Rd1 mouse. Histological and electroretinogram analyses were performed to evaluate the retina integrity. At P12, an early stage of the disease, Rd1 mice displayed an increased expression of Cdk4 and Cdk2, as well as their target, phosphorylated-Rb among photoreceptor nuclei. Genetic and pharmacological interferences with cell cycle protein activities extended photoreceptor survival both in vitro and *in vivo*, but transiently. Interestingly, we observed that at P12, the polycomb group protein Bmi1 was expressed in virtually all the nuclei in the inner and outer nuclear layer of both wild-type (WT) and Rd1 mice. Bmi1 promotes cell cycle progression via the repression of tumor suppressor genes through epigenetic modifications. We reasoned that Bmi1 deletion could impede the Cdk reactivation that characterizes neuronal apoptosis and may therefore delay retinal degeneration. We compared the histology of WT, Rd1 and Rd1;Bmi1-/- and observed the presence of 7 rows of photoreceptors in Rd1;Bmi1-/- mice at P33, while Rd1 littermates displayed a single scattered row of photoreceptors. ERG recordings revealed the ability of Rd1;Bmi1-/- retinas to respond to light stimuli (cone response). Cdk4 activity and cell death were strongly decreased in Rd1;Bmi1-/- mice, respectively by 70% and 50% as compared to Rd1 littermates. Both rods and cones were well preserved in the Rd1;Bmi1-/- retinas. In conclusion, our data show for the first time a mechanism of retina degeneration involving a reactivation of the cell cycle that precedes photoreceptor death. Moreover, Bmi1, known to play a key role in the control of neural stem cell renewal, appears also to have a permissive action on neurons to allow cell cycle entry and progression until the S-phase. The dissection of such mechanisms may help to understand cues controlling cell regeneration versus cell death.

Poster Board Number: T-1012

STARGARDT'S DISEASE: TOWARD AN *IN VITRO* MODEL OF THE MACULAR DYSTROPHY.

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Stargardt's disease is a genetic autosomal-recessive disorder with the prevalence rate 1 in 8-10 thousand people. The hereditary disease leads to loss of central vision starting from first or second decades of life due to a macular degeneration. One of clinical manifestations of the condition is presence of lipofuscin granules - yellowish autofluorescent flecks containing A2E and its derivates. These components are toxic and their accumulation is believed to result in death of retinal pigment epithelium (RPE) cells and consequent loss of photoreceptors. Mutations of ABCA4/ABCR are accountable for most cases of Stargardt's macular dystrophy. The ABCA4 transporter defects impair 11-cis-retinal cycle (visual cycle) causing the accelerated accumulation of A2E and its derivates. Noteworthy, A2E and its derivates accumulation occurs also in other diseases including age-related macular dystrophy and gradually develops during lifetime of healthy people. Therefore, significance of development of Stargardt's disease in vitro model, which is the purpose of our study, goes beyond studying of mechanisms of and developing treatments for the particular macular dystrophy. Obtaining of the in vitro model will also facilitate study some other macular degeneration diseases and normal aging process in human eyes. We have successfully obtained and characterized induced pluripotent stem (iPS) cells from skin fibroblasts of 2 siblings with Stargardt's disease. We developed a robust protocol for *in vitro* differentiation of hESCs and iPSCs into three-dimensional structures histologically and molecularly similar to developing retina. Using 3-step differentiation protocol combining 2D and 3D cultivation of differentiating hESCs and iPSs we induced "eye-like" structures containing neural retina surrounded by retinal pigmented epithelium layer. Histological, immunochemical, and PCR analysis demonstrated that "3D eye-like" structures derived from hESCs, iPSCs from normal donors and patients with Stargardt's disease closely reminded flatten pattern of natural eye development. No evidence has been published for the existence of the visual cycle in culture of human retinal cells. This cycle implies contact and proper functional interaction between photoreceptors and RPE cells. Since defects in the 11-cis-retinal cycle lead to the pathology then obtaining of retinal cells culture, where the visual cycle occurs, is one of the most important steps in developing in vitro model of Stargardt's disease. We are currently addressing the question whether the 11-cis-retinal cycle can be observed in our cultures of retinal cells.

Poster Board Number: T-1013

ROLE OF GLAST-POSITIVE GLIAL CELLS DURING PHOTORECEPTOR DEVELOPMENT

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Taking advantage of two transgenic mouse lines, glast.dsRed and crx.gfp, that express fluorescent proteins in glial and photoreceptor cells respectively, we investigate the role of glast-positive glial cells (GPCs) in the survival/differentiation/proliferation of age-matched and aged photoreceptor cells. Primary retinal cells were isolated from newborn transgenic mouse retina (glast.dsRed::crx.gfp) at postnatal day (P0/P1) and propagated in defined medium containing epidermal growth factor (EGF) and fibroblast growth factor 2 (bFGF). By flow-sorting another population of pure GPCs was purified. Both populations were expanded and analyzed for the presence of specific retinal cell markers. Notably, the primary cell culture collected from the glast.dsRed::crx.gfp transgenic line showed a conspicuous presence of immature photoreceptors growing on top of GPCs. In order to reveal the role of such cells in the survival/differentiation/proliferation of photoreceptors we set up in vitro cultures of retina-derived cells that allowed long-term time-lapse recordings charting every cell division, death and differentiation event. To assess the regenerative potential of cultured GPCs, we challenged them with compounds mimicking retinal degeneration (NMU, NMDA, Zaprinast). Moreover, in order to evaluate

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the ex-vivo capability of GPCs to generate/regenerate photoreceptors, we set up a time-lapse video of glast.dsRed::crx.gfp-positive retinal explants in wt or degenerating conditions at different developmental stages. Mass spectrometry (MS), immunostainings and other molecular approaches were performed to reveal adhesion molecules involved in the relationship between glial cells and photoreceptors. Both primary cell lines were highly homogenous, with an elongated morphology and the majority expressed Müller glia markers (MG) such as glast, blbp, glt-1, vimentin, glutamine synthetase (GS), GFAP, cd44, mash1 and markers of reactive Müller glia such as nestin, pax6. Conversely, none of them were found positive for retinal neuron markers like tuj1, otx2, recoverin.Primary cultures of GPCs show the incapability of glial cells to give rise to photoreceptors in both wild type or degenerative environment. Furthermore, primary cultures of pure GPCs challenged with different compounds did not highlight the production of new glial cell-derived photoreceptors. Nonetheless, GPCs support photoreceptor survival for more than 30 days. Retinal explants treated with compounds causing retinal degeneration *in vivo*, show interesting results that are still under analysis. Adhesion molecules involved in the contact between photoreceptors and glial cells are under investigation. In conclusion, primary glial cell cultures collected at P1/P0 do not give rise to photoreceptor cells in wt or degenerative conditions at least in vitro. Their role in ex-vivo and in-vivo experiments in both wt or degenerative conditions are still under further investigations. Presently, data obtained in culture strongly suggest that glial cells control the survival of photoreceptor cells.

Poster Board Number: T-1014

EVALUATION OF CROSS-LINKING TIME FOR CARBODIIMIDE MODIFIED AMNIOTIC MEMBRANES ON LIMBAL EPITHELIAL CELL CULTURE CHARACTERISTICS

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Purpose: This study aims to investigate the effect of cross-linking time for carbodiimide modified amniotic membrane (AM) on limbal epithelial cell (LEC) culture characteristics. Methods: The AM was chemically cross-linked with 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC)/N-hydroxysuccinimide (NHS) for a variety of time periods in the range of 1-48 h. Ninhydrin assays and mechanical tests were used to determine the extent of cross-linking. In addition, the degradability was studied by incubating the AM at 37°C in balanced salt solution containing collagenase. After cultivation on various cross-linked AM samples, the human LECs were analyzed for their proliferation and gene expressions. Results: The results of ninhydrin assays and mechanical tests showed that an increase in the cross-linking time may lead to the increase in the cross-linking degree of chemically modified AM. After treatment with EDC/NHS for 48 h, the samples had strong mechanical strength and exhibited good degradation resistance, indicating that these biological tissue materials could provide stable support for LEC growth. It was found that the cells on the AM modified with carbodiimide chemistry for a longer time expanded rapidly and expressed higher levels of p63 and ABCG2. Conclusions: The present study suggests that the cross-linking time has a profound influence on the performance of carbodiimide modified AM for LEC cultivation.

Poster Board Number: T-1015

DEVELOPMENT OF PATIENT SPECIFIC IPS CELLS IN ORDER TO MODEL LEBER CONGENITAL AMAUROSIS DISEASE

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Retinitis pigmentosa (RP) and Leber Congenital Amaurosis (LCA), a genetically heterogeneous group of diseases, together with age-related macular degeneration (AMD) are the leading causes of permanent blindness characterized by progressive degeneration of photoreceptors and pigment epithelial cells of the retina. The aim of this study is to develop retinal progenitors from patients-own somatic cells that will serve as an experimental model of the disease and eventually applied in clinical therapy to reverse blindness. Patients' dermal fibroblasts, obtained by punch biopsy, have been selected as a source of retinal progenitors. The fibroblasts were de-differentiated into a pluripotent stage (human induced pluripotent stem, hiPS, cells) by retroviral particles producing hNANOG, hOCT4, hSOX2, hKLF4 and hc-Myc produced in HEK293T cells. The colonies were propagated and characterized for pluripotency imunocytochemicaly and by RT-PCR. The identity of the derived iPS cells was confirmed by fingerprinting. Early-onset RP patients' fibroblasts with mutations in *CRB1* (W822STOP/C948T) and *RDH12* (T49M) genes were reprogrammed into hiPS cells. 21 days after transduction colonies with the morphology similar to embryonic stem cells begun to appear. The colonies were similar to healthy patient hiPS colonies as characterized for pluripotency by immunocytochemistry and RT-PCR. We successfully generated RP patient-specific iPS cell lines and characterized them for pluripotency. These cells are being submitted to the differentiation protocol to generate fotoreceptor and retinal pigment epithelial cells. The use of patient derived iPS cells will help to elucidate the pathogenic mechanism caused by the mutations in *CRB1* and *RDH12* genes.

Neural Cells

Poster Board Number: T-1016

CTX HUMAN NEURAL STEM CELL (HNSC) THERAPY FOR ISCHEMIC STROKE PATIENTS: PISCES - A CLINICAL SAFETY TRIAL

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Background: The hNSC line CTX0E03 was clonally derived from early expansion of fetal cortical hNSCs following retroviral insertion of a c-mycERTAM fusion gene that permits controlled expansion in the presence of growth factors and 4-hydroxytamoxifen. The CTX0E03 cell line has been banked and is used in the GMP (Good Manufacturing Practice) manufacture of CTX Drug Product (DP) currently in clinical development. Preclinical studies with CTX DP in MCAo (Middle Cerebral Artery Occlusion) rat stroke models have shown dose-related behavioral recovery in sensorimotor function and evidence of cell survival, limited migration, angiogenesis & endogenous neurogenesis. In the PISCES trial we aim to investigate the safety profile of intra-cerebral implantation of CTX DP; a secondary aim is to explore indices of change in neurological function as a result of cell treatment. Methods: We undertook an open label,

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ascending dose, Phase I clinical trial in 12 male patients with stable neurological deficit & disability resulting from ischemic stroke affecting the basal ganglia or white matter 6 months to 5 years previously. Four groups of 3 patients receive 2, 5, 10 & 20 million cells respectively, implanted in the ipsilateral putamen by stereotaxic injection. The two year follow-up includes a range of neurological function tests as well as general clinical & longitudinal radiological data (3T MRI, fMRI, DTI, MRS) to identify potential biomarkers of change in neurological function. FMRI data collection pre & post cell implantation is based on a block design of hand grasp-release task that is visually cued at 0.25Hz. All patients will be followed up for 10 years and give consent for post-mortem brain examination. Results: Up to January 2012, five male subjects had been implanted with CTX0E03 [2 million (n=3) & 5 million (n=2) cells] with follow up between 2 and 14 months. Mean age was 74.6 years (range 68-83) with median National Institutes of Health Stroke Scale (NIHSS) score of 8 and mean time from stroke onset 30.2 months (range 14-51). Two (40%) had sub-cortical-only infarcts and 3/5 (60%) had left hemisphere lesions. No direct cell related adverse events or immune related response have been observed. One patient had a minor procedure-related asymptomatic sub-dural bleed & 2 had superficial scalp infection at the wound site. NIHSS scores 1 month post cell implantation improved in all 5 patients with reductions in score compared to baseline being 2, 1, 3, 1 & 1 respectively and further reduction by 1 & 2 at 12 month review in the first 2 patients. Summated Ashworth spasticity scores of affected limbs (total potential score=60) improved in all 5 patients compared to baseline by 15, 7, 6, 3 & 8 respectively. Comparing fMRI activations (SPM8; active vs rest; $p<0.05$) in 2 subjects with hand weakness but with 1 preserved & 1 damaged motor cortex shows widespread activations followed by reorganization to ipsilesional cortex during return of minimal finger movement and bilateral occipital & cerebellar activation followed by return of surviving ipsilesional cortex activation during minimal leg power improvement, respectively. Conclusions: No cell related adverse events observed to date in the first 5 patients enrolled. Neurological impairment improved in all subjects, and was sustained in longer-term follow up. Moreover, some longitudinal change in motor activation fMRI was seen. The PISCES trial will continue to enroll subjects at the remaining and higher dose cohorts according to ongoing data review.

Poster Board Number: T-1017

CELLULAR NEURODEGENERATIVE DISEASE MODELS DERIVED FROM GENETICALLY MANIPULATED HUMAN PLURIPOTENT STEM CELLS

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Cellular disease models derived from human pluripotent stem cell (hPSC) are demanded in research of disease mechanisms as well as screening of new drugs because animal models or tumor cell-derived models do not always precisely mimic human responses. Recently we have established an efficient system to integrate genes into a defined locus in hPSC. Using this site-specific gene integration system, we have generated hESC and hiPSC lines carrying mutant genes responsible for familial neurodegenerative diseases, such as Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), and Huntington's disease (HD). Cellular disease-phenotypes were detected in the disease model cells. For example, hESC-derived AD models showed AD-related phenotypes such as increase of Aβ42 production and neural dysfunction. HD models showed cell death of mutant HTT-expressing neurons in particular conditions. In addition, our disease models derived from genetically manipulated hPSCs are capable of comparing with isogenic healthy hPSCs. These results indicate that genetically manipulated hPSC-derived disease model cells are useful tools for neurodegenerative disease research.

Poster Board Number: T-1018

LONG-TERM STABLE *IN VITRO* CULTIVATION OF NEURAL STEM CELL LIKE CELLS FROM HUMAN ADULT TEMPORAL LOBE

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Autologous adult human neural stem cells (ahNSCs) may be used for regenerative cell therapies bypass potential ethical problems. However, stable *in vitro* expansion protocols and experimental/clinical factors influencing primary cultures need to be further elucidated for clinically applicable techniques. To address these issues, we obtained biopsy specimens from 23 temporal lobe epilepsy patients. Neural cells were primarily cultured in anewly defined attachment culture condition and experimental/clinical factors influencing the culture were analyzed. When the success of primary cultures was defined as stable expansion of cells (>ten *in vitro* passages) and expression of NSC markers, success rate of the primary culture was 39% (nine of 23 temporal lobes). In the successful cases, 1×10^5 neural cells could be multiplied into 1×10^{15} cells within four months. During the long-term expansion, expressions of NSC markers and differentiation potentials into astrocytes and neurons were maintained. After the 18th sub-culture, spontaneous senescence and differentiation were observed, and the cultivated NSC-like cells ceased their proliferation. The culture results were not affected by seizure characteristics. However, an older age (> 40 years) and a smaller sample volume (< 2ml) were found to exert negative influences on the primary culture results. Although NSC-like cells were obtainable from the lateral temporal lobe remote from the subventricular zone, they were more successfully cultured from the medial temporal lobe facing the ventricular surface. The results here would provide promising experimental and clinical strategy of using patient-specific autologous NSCs in regenerative medicine in the future.

Poster Board Number: T-1019

TRANSPLANTATION OF NEURAL CELLS DERIVED FROM HUMAN IPS CELLS INTO AN ALZHEIMER'S DISEASE MODEL

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Aim: Alzheimer's disease (AD) represents a major cause of early-onset and progressive dementia and is characterized with degeneration of cholinergic neurons especially in the hippocampus. Radical cures have not been developed at present. Amyloid precursor protein (APP) mutation causes familial AD and several APP isoforms are suggested to be associated with AD. In this study, we tried to generate cholinergic neurons from human iPS (hiPS) cells and to transplant the cells to the bilateral hippocampal lesions of APP

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transgenic mice. Methods: (Culture of hiPS cells) A hiPS cell line was obtained from RIKEN Cell Bank (cell name: 253G1, cell number: HPS0002) and was maintained according to RIKEN cell preparation manual. (Cell differentiation) hiPS cells were maintained in the presence of mouse embryonic fibroblasts cells in serum-free medium containing basic fibroblast growth factor. We developed embryonic bodies (EBs) from undifferentiated hiPS cells by 4-day floating culture. EBs were transferred to fibronectin-coated dishes and cultured for 24 hours. We added retinoic acid, sonic hedgehock and noggin in the dishes twice and cultured for 72 hours. We conducted RT-PCR analysis and immunocytochemistry to evaluate hiPS cell differentiation. (Transplantation) The burr hole marks were made at 2.0mm lateral and 2.4mm posterior to the bregma in bilateral parietal bones. The hiPS cells derived neural cells were injected into the bilateral hippocampal lesions (1.75mm depth from the brain surface) of two-month-old APP transgenic mice. Learning and memory functions were evaluated by Morris water maze before and after the transplantation. Histology and immunohistochemistry were performed six months after the transplantation. Results: (Cell differentiation) The cells derived from hiPS cells expressed neural markers in RT-PCR and confocal immunofluorescence analysis as well. The former showed that the cells expressed genes of chorine acetyl transferase, beta III tubulin, nestin, neurofilament and Islet1. The latter disclosed that the cells were 30-50% neural cell adhesion molecule (NCAM) positive, 90% nestin positive and 90% beta III tublin positive. (Transplantation) Transplanted hiPS cell derived neural cells were localized in the bilateral hippocampal lesions and these cells expressed human NCAM. There were no significant differences in the results of Morris water maze between hiPS cell and vehicle transplanted transgenic mice at this time but remarkable improvement of performance in this test was achieved in several hiPS cell derived neural cell transplanted mice. Conclusions: We established a model of bilateral neural transplantation for AD and the behavioral assessment was improved in several APP transgenic mice. We speculate that hiPS cell derived neural cell transplantation to the hippocampus is a promising candidate for AD treatment.

Poster Board Number: T-1020

TWO FACTOR REPROGRAMMING OF PERICYTE-DERIVED CELLS OF THE ADULT HUMAN BRAIN INTO FUNCTIONAL NEURONS

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Direct conversion of somatic cells into neurons provides a new approach towards cell-based therapy of neurodegenerative diseases. Recent studies have shown that the synergistic action of 3-4 transcription factors can induce neurogenesis from fibroblasts as well as hepatocytes which represent a non-ectodermal cell population. However, a major challenge for the translation of neuronal reprogramming into therapy concerns the question whether the adult human brain itself contains cell populations amenable to direct somatic cell-to-neuron conversion. Here we show that cells from the adult human cerebral cortex expressing pericyte markers, such as PDGFRβ, can be reprogrammed into functional neurons by retrovirus-mediated co-expression of only two transcription factors, namely Sox2 and Mash1. These MAP2- and NeuN-expressing neurons acquire the ability of repetitive action potential firing and serve as synaptic targets for other neurons indicating their capability of integrating into neural networks. The pericytic origins of those neurons was verified by PDGFRβ-based sorting of the cells and subsequent continuous live imaging during reprogramming. Furthermore, genetic fate-mapping in mice expressing an inducible Cre recombinase under the tissue nonspecific alkaline phosphatase promoter corroborated the pericytic origin of the reprogrammed cells. Our results demonstrate that cells endogenous to the adult mouse and human brain can be directly converted into functional neurons by only two transcription factors representing a viable approach for cell-based therapies of neurodegenerative diseases.

Poster Board Number: T-1021

MODELING INFLAMMATION IN PARKINSON'S DISEASE USING HUMAN PLURIPOTENT STEM CELLS

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Parkinson's Disease (PD) is the most common neurodegenerative movement disorder. Historically, PD has been considered a strictly neuronal disease; however, clinical observations and evidence from animal models suggest inflammation may contribute to disease progression. It remains controversial whether glial activation, and the resulting inflammatory cascade, is a result or a cause of neuronal death. Towards resolving this distinction, we have established cultures of human embryonic stem cell derived dopaminergic neurons, human astrocytes and microglia. These neural cells are used to investigate the inflammatory response to alpha-synuclein, the protein whose mis-folding is the pathological hallmark for all forms of PD. We found alpha-synuclein is sufficient to induce the secretion of pro-inflammatory cytokines in both human astrocytes and microglia, and established the neurotoxic response to this glial derived increase in pro-inflammatory cytokines, allowing us to study the inflammatory contribution to the pathological development of PD in a human system. Furthermore, we are reprogramming fibroblasts from patients with idiopathic PD to investigate the contribution of inflammation to the progression of non-genetic forms of this disease. We aim to understand the role of inflammation in PD and identify key molecular events involved in preliminary stages of PD to exploit as potential targets for therapeutic intervention early in the course of the disease.

Poster Board Number: T-1022

A NEW TYPE OF HUMAN NEURAL STEM CELL FOR EFFICIENT PRODUCTION OF OLIGODENDROCYTE-LINEAGE CELL

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Oligodendrocytes are known to play a critical role in the pathogenesis of many neurological disorders including spinal cord injury, multiple sclerosis, schizophrenia, and congenital demyelinating diseases. A re-myelination strategy for spinal cord injury is well established and several treatments using neural stem cell/oligodendrocyte progenitor cell have undergone preclinical studies and/or clinical trials [i.e. human embryonic stem cell (HESC)-derived

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oligodendrocyte progenitor cell (OPC) (Geron Corp., terminated Phase I trial), human neural stem cell (NSC) (Stem Cells, Inc., Phase I clinical trial in Switzerland) (Neuralstem, Inc.), autologous schwann cell (Miami project) and autologous bone marrow-derived cell (SanBio, Inc.)]. However, there are pros and cons for these cells in term of efficiency. NSCs seem to have higher survival rate and migration potential than other cells but other cells have higher differentiation potency into myelin-forming cells. Human neural stem cells (hNSCs) are usually cultured in a floating condition, called neurosphere culture, to maintain their multi-potential and proliferation potential. However, neurospheres contain many different progenitors and differentiated cells and the ratio of neural stem cells is usually very low. Recently, hNSCs could be expanded under adherent monolayer culture condition on laminin-coated cultureware in the presence of EGF and bFGF. However, their differentiation potential into oligodendrocyte was not so high. I developed a new adherent monolayer culture method that enables hNSCs to grow exponentially and to differentiate into oligodendrocyte-lineage cells at 99% efficiency. Human NSCs dissociated from human fetal tissue were initially cultured in an ordinary chemically-defined culture medium in the presence of growth factors. The cells could be maintained but cell number decreased gradually after several passages. At passage 7, the cells were cultured in a proprietary NSC medium. While the cells couldn't proliferate and total cell number was decreased in an ordinary culture medium, they could proliferate and be successfully expanded 10,000,000 times after another 12 passage in the proprietary medium with the same growth factor combination. The cells displayed a homogeneous morphology and most of them expressed CD133 (100% by flow cytometry), Sox2 and nestin. When they were differentiated by withdrawing growth factors in a serum-containing medium, neuron (ßIII-Tublin positive-, Neurofilament L positive - and/or MAP-2-positive cells), astrocytes (GFAP-positive cells), and oligodendrocytes (O4 positive -, GalC positive - and/or MBP positive - cells) could be observed. These data indicate that they retain neural stem cell characteristics under this culture condition. When they were differentiated in a serum-free condition, 23.5 ± 2.0% of total cells seemed to retain undifferentiated state and 99.1 ± 0.6% of differentiated cells (75.8 ±2.1% of total cells) showed pro-oligodendrocytes (O4-positive/GalC-negative) or oligodendrocytes (O4-positive/GalC-positive) phenotype. This result suggested that the new culture condition shifted the cell fate to oligodendrocyte-lineage even though they retained their neural stem cell characteristics. Furthermore, this method will enable production of human oligodendrocyte-lineage cells in numbers sufficient for cell therapy.

Poster Board Number: T-1023

SORTING AND TRANSPLANTATION OF DOPAMINERGIC PROGENITOR CELLS DERIVED FROM HUMAN PLURIPOTENT STEM CELLS

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Cell grafts in clinical use should be not only free from animal-derived factors or feeder cells, but also free from graft-derived tumors due to contamination of undifferentiated or unwanted cells. In this report, we examined a nearly xeno-free culture method with FACS-based sorting using a surface marker of the floor plate cells, to purify the ideal cell graft for Parkinson's disease. We induced neural progenitors from human ES cells (KhES-1) and human iPS cells (404C2) by the SFEBq (serum-free embryoid body quick) method with small molecules BMP inhibitor and Activin/Nodal inhibitor to induce neural cells efficiently, and we analyzed the expression of a cell surface marker Corin, by qPCR and flow cytometry. By the SFEBq method, expression of Corin arises from culture day 10 and about 10-20% of living cells were positive on day 21. After sorting, purity of Corin positive cells were over 90%, and the purity of the cells were confirmed by qPCR analysis. Corin positive cells were also expressed Lmx1a, a transcript for midbrain dopaminergic progenitors. Corin positive cells differentiate to TH positive neurons after 2-3 weeks of *in vitro* culture, and the TH positive cells were also immunoreactive for Nurr1 or Pitx3, as a marker for midbrain dopaminergic neurons. When grafted into non-lesioned NOD-SCID mice on 3 days after cell sorting (differentiation totally for 24 days), no tumor was found in the graft of Corin positive cells, while graft overgrowth was found in the graft of Corin negative or unsorted cells, and more TH+ neurons were survived in Corin positive graft. After modification of the culture methods and the timing of sort, we grafted the Corin positive cells into the 6-OHDA lesioned rats. Human cells-derived TH positive cells were found in the graft for 8 weeks after transplantation, and methamphetamine-induced rotation behavior were partially improved in the grafted animals with "proper" midbrain dopaminergic neurons. We succeeded in cell sorting for dopaminergic progenitors using a surface marker Corin, and dopaminergic neurons were survived and functioned in the 6-OHDA rats. Sorting of dopaminergic progenitor cells using a surface marker reduced the risk of graft overgrowth and functioned in rodents. We are planning transplantation of the sorted cells to MPTP-lesioned monkeys to make a functional evaluation.

Poster Board Number: T-1024

AN *IN VITRO* MODEL OF THE HUMAN REGIONALISED BRAIN REVEALS VENTRAL-TO-DORSAL FATE SWITCHING UPON TRANSGENIC EXPRESSION OF THE TRANSCRIPTION FACTOR LMX1A

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Detailed studies of fate-determining genes in brain development have so far been limited to rodents and other smaller animals, in which it is possible to study the effect of transgenesis in the context of the intact developing brain. In contrast, the importance of fate-determining genes in human brain development remains largely unexplored due to the lack of appropriate model systems. Here, we present a system for studying region-dependent neural fate switches in an *in vitro* model of the developing human brain by the use of human embryonic stem cells (hESCs). Through dose-dependent inhibition of glycogen synthase 3 (GSK3), we activated WNT signalling and induced gradual caudalisation of our hESC-derived neural progenitors to yield cell fates ranging from telencephalic to posterior hindbrain fates. The authenticity of our regionalized cultures was verified by parallel gene expression analyses in sub-dissected tissue from the human fetal brain. In rodents, the transcription factor Lmx1a has been shown to be involved in the development of mesencephalic dopaminergic (mesDA) neurons, but also in the formation of roof plate, cerebellum and choroid plexus. For studying fate-determining effects of Lmx1a in human cells, we used a lentiviral system under control of the pluripotency-associated microRNA mir292 to ensure transgene expression only in the differentiated cell population and not in the pluripotent hESC population. We found that transgenic Lmx1a did not affect neuralisation of the cells, nor the rostro-caudal identity of the neural progenitors. However, at all levels of the neural axis, Lmx1a suppressed formation of ventral and floor plate fates and instead caused induction of dorsal and roof plate fates, which was

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evident as a strong activation of Msx1, Pax6, Pax7, Gdf7 and Wnt1. In forebrain and hindbrain cultures, but not in midbrain cultures, Lmx1a induced formation of choroid plexus, in accordance with the anatomical derivation of choroid plexus from forebrain and hindbrain roof plate. Transgenic expression of Lmx1a in ventralised midbrain cultures suppressed FoxA2 expression and abolished the formation of mesDA neurons, which were otherwise generated in large numbers from these cultures. In contrast, Lmx1a induced the formation of tyrosine hydroxylase (TH) expressing neurons from telencephalic cultures at the expense of ganglionic eminence fates. These Lmx1a-induced TH+ neurons did not co-express FoxA2 or the enzyme AADC, and thus did not represent mesDA neurons. In conclusion, we have developed a system for studying context-dependent effects of neural fate determinants in human cells. We found that transgenic expression of Lmx1a induced dorsalisation of human neural progenitors and caused formation of region-specific dorsal-derived fates, but did not contribute to formation of mesDA neurons in our system.

Poster Board Number: T-1025

ELUCIDATING THE ROLE OF LYSOSOMAL DYSFUNCTION IN THE PATHOLOGY OF PARKINSON'S DISEASE USING HUMAN ADULT INDUCED PLURIPOTENT STEM CELLS DERIVED FROM PATIENTS

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Parkinson's Disease (PD) is a common neurodegenerative disease, affecting more than 1% of the aged population. The identification of several genetic mutations associated with PD has lead to the discovery of important pathological mechanisms, which now have the potential to help better understand the pathology of most common idiopathic forms of PD. Recent clinical evidence has shown an association between mutations in glucocerebrosidase (GBA) and PD. In fact, heterozygous GBA mutations represent the highest risk factor for PD. Accumulation of misfolded proteins is a common pathology in PD, and GBA could be important for protein degradation via the lysosomal pathway. We aim to elucidate the role of heterozygous GBA mutations in the pathology of PD, by focusing on lysosomal dysfunction in the context of autophagy and translate these same mechanisms into the context of idiopathic PD. The Oxford Parkinson's disease Centre has screened around 600 patients for two common GBA mutations (N370S and L444P). Positive cases (11 N370S and 6 L444P) have been identified by specific restriction enzyme digestion and sequence analysis. Several idiopathic PD patients and age matched healthy controls have also been recruited for this project. Neuronal cultures derived from induced pluripotent stem cells (iPSC) generated from PD patients potentially offer unique insights into the dysfunction of the dopamine neuronal network which occurs in PD. Therefore, PD (idiopathic and GBA carriers) and control iPSCs have been generated by retroviral delivery of reprogramming factors (Oct3/4, Sox2, c-Myc, Klf4 and Nanog) to primary dermal fibroblasts. We have optimized an efficient protocol for the specific differentiation of dopaminergic neurons from iPS cells. Initially, SMAD signalling inhibition (Noggin and SB431542) was applied, followed by neuralisation using SHH, AA, BDNF and FGF8. Final midbrain maturation was obtained by the addition of cAMP and GDNF. Neurons developed a mature neuronal morphology, with the majority expressing neuronal markers (TuJI, MAP2, NeuN). Further characterization showed a consistent high expression of TH and other relevant midbrain markers (eg. DAT, En1, Nurr1, Pitx3 and FoxA2). Functional dopamine transporter was confirmed by 3HDA uptake assay, and production of dopamine was confirmed by HPLC analysis. Electrophysiology studies also confirmed efficient electrical activity by these cells, presenting a healthy negative resting membrane potential, Na+ and K+ channel activity and development of action potentials when stimulated. Using this stem cell based model we have confirmed the dopaminergic identity of the differentiated cells obtained, and have been working on functional comparison between idiopathic and age-matched control focusing initially on dopamine homeostasis. Potential lysosomal deficits, which may be impaired in both GBA and idiopathic DA neurons, are currently under investigation, looking into levels of lysosomes and autophagosomes production, fusion and transport. Common molecular pathways are likely to be involved in the neurodegenerative process of diverse PD cases, where this relevant human neuronal stem cell based model might be central to understanding the early pathology of PD.

Poster Board Number: T-1026

SMALL MOLECULES ENABLE HIGHLY EFFICIENT NEURONAL CONVERSION OF HUMAN FIBROBLASTS

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Advances in transcription factor-based cell reprogramming of somatic cells into pluripotent and alternative somatic fates have changed our view of a unidirectional way of cell specification. Results of recent studies indicate that murine and human cells from the mesodermal lineage (fibroblasts) can be directly converted to an ectodermal lineage (neurons) with a cocktail of defined transcription factors. Since neurons are postmitotic, the conversion efficiency represents an important parameter for subsequent biomedical applications such as disease-modeling or neuroregenerative therapies. We set out to explore whether the efficiency of converting human postnatal fibroblasts (HPF) into induced neuronal-like cells (iNs) can be enhanced by small molecules in combination with a minimum number of transcription factors. To that end we combined two factor neuronal programming with small molecule-based inhibition of WNT and BMP signaling. Thereby we were able to generate cells exhibiting morphological, immunocytochemical and functional properties of postmitotic neurons of different neurotransmitter phenotypes with an up to >17fold increase in conversion frequency. Specifically, we could generate iNs with conversion yields exceeding 200% and final neuronal purities of up to >80%. Our data demonstrate that the generation of iNs from HPF can be significantly enhanced using a cocktail of neural inducing factors. We expect this experimental approach to be suitable for the direct generation of bulk quantities of human iNs for disease modeling and other biomedical applications.

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Poster Board Number: T-1027

DEVELOPMENT OF A HUMAN EMBRYONIC STEM CELL-BASED DISEASE MODEL FOR AMYOTROPHIC LATERAL SCLEROSIS.

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Generation of amyotrophic lateral sclerosis (ALS) disease models is an important subject for investigating disease mechanisms and pharmaceutical applications. Transgenic mice expressing mutant form(s) of superoxide dismutase (SOD1) can lead to the development of ALS that closely mimics the familial type of the ALS (FALS). Although SOD1 mutant mice show similar phenotypes to FALS, the difference of species, dissimilar drug responses as well as the size differences limit their usefulness to study the mechanism(s) and identify potential therapeutic compounds. Development of an in vitro model system for ALS is therefore expected to overcome some of the above limitations and help in obtaining novel insights into disease mechanisms and discovery of therapeutics. We established an in vitro FALS model from human embryonic stem cells (hESCs) expressing either a Wild Type (WT) or a mutant SOD1 (G93A) gene and evaluated the phenotypes of the spinal motor neurons (sMN), which are the affected neurons in ALS patients, as well as their susceptibility to degeneration. Transgenic hESC clones were generated by transfection of vectors expressing either a WT or a G93A SOD1 mutant gene. Spinal motor neurons were obtained by a differentiation protocol described earlier [1]. Cell biological assays were performed to evaluate the in vitro FALS model. The in vitro model we developed mimics the in vivo human ALS disease in terms of the 1. Selective degeneration and cell death of spinal motor neurons expressing the G93A SOD1 but not those that express the WT gene, 2. susceptibility of SOD1-derived sMN to form ubiquitin aggregates. We also show the involvement of astrocyte-derived factor(s) in the selective degeneration of G93A SOD1 sMN. Thus, this model is expected to help unravel to disease mechanisms involved in the development of FALS and also lead to potential drug discoveries based on the prevention of neurodegeneration
References: [1] Wada T et al: Highly efficient differentiation and enrichment of spinal motor neurons derived from human and monkey embryonic stem cells. PLOS one, 4(8), e6722, 2009.

Poster Board Number: T-1028

OPTIMIZATION OF NEURONAL CULTURES DERIVED FROM HUMAN INDUCIBLE PLURIPOTENT STEM CELLS FOR HIGH TRHOUGHPUT FUNCTIONAL SYNAPTIC ACTIVITY ASSAY

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Background: We previously described the development of a high-throughput screening technology for identifying modulators of synaptic function, termed the MANTRA™ (Multiwell Automated NeuroTRansmission Assay) system (Hempel CM et al., 2011). We have demonstrated the utility of the MANTRA system for performing assays of synaptic vesicle cycling in rodent primary neuronal cultures in 96-well plates. Here, we show that cultures of human neurons derived from induced pluripotent stem cells (iPSCs) can be utilized in the MANTRA system for synaptic functional assays. Results were compared to those generated from rat primary neuronal cultures. Methodology: Post-mitotic human neurons derived from iPSCs ("iCell® Neurons", Cellular Dynamics International, USA) and primary neuronal cultures isolated from rat embryos were seeded in 96-well plates (Greiner) coated with poly-D-lysine with or without laminin. For some experiments, iCell Neurons or rat neurons were cultured with astrocytes grown as a monolayer. iCell Neurons and rat neuronal cultures were seeded on the same plates and tested in parallel. For analysis of ability of neurons to initiate action potentials following field stimulation, neurons were loaded with Fluo-4. For analysis of presynaptic function, cultures were infected with an adeno-associated virus (AAV) used to deliver a synaptophysin-pHluorin fusion fluorescent reporter construct (sypHy). Cultures were analyzed between 2 and 7 weeks *in vitro* on the MANTRA system or on a fluorescence microscope imaging system. For both systems, fluorescence imaging was performed in parallel with field stimulation trains. Immunofluorescence analysis was performed at different time points to evaluate the expression and localization of presynaptic proteins and the sypHy reporter. Results: We found that iCell Neurons exhibit expected neuronal and synaptic morphology and can be reliably transduced with AAV to express sypHy as early as 2 weeks *in vitro* with no signs of cytotoxicity. Immunostaining analysis revealed that sypHy is expressed in iCell Neurons in a punctuate pattern and is co-localized with synapsin I. iCell Neurons showed robust Ca2+ responses to field stimulation trains with voltage thresholds similar to those of rat neurons, suggesting reliable generation of action potentials. iCell Neurons at 3-6 weeks *in vitro* displayed measureable levels of evoked presynaptic activity on the MANTRA system. Follow up high-resolution microscope analysis confirmed the synaptic localization of sypHy signals in iCell Neurons. Although pre-synaptic responses were lower in iCell Neurons than in rat neuronal cultures several conditions were found to increase the response levels. Treatment of iCell Neurons for 1 hour with a compound known to increase evoked pre-synaptic activity yielded similar presynaptic effects on human iCell Neurons and rodent primary neuronal cultures. Also, co-culturing of iCell Neurons with glia resulted in a robust increase in evoked presynaptic responses. Conclusions: The MANTRA system can measure synaptic activity in human neurons derived from iPSCs. The high-throughput capacity of this system provides a unique capability to test multiple conditions in parallel to generate human iPSCs-derived neurons with optimal synaptic functionality. Ultimately, the MANTRA system can be used to characterize synaptic abnormalities in neurons derived from patients and to screen for compounds to restore normal synaptic transmission.

Poster Board Number: T-1029

CD81 IS HUB REGULATOR IN HUMAN NEURAL STEM CELL MIGRATION

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Tetraspanin proteins have been implicated in many cellular activities, including adhesion, migration, and proliferation, however their exact function is unknown. Specifically, CD81 (TAPA-1) has been involved in cell migration. But mechanisms are poorly defined in any of stem cells. We describe a novel mechanism whereby the CD81 can affect cell motility and induce migration onto ECM substrates in HNSC(Human Neural Stem Cell). RT-PCR and Western-blot analysis confirmed CD81 expression in HNSC. Immunoprecipitation analysis confirmed CD81 interactions with MMP-9 and integrin beta 1 in HNSC. Also, the expression of the CD81, MMP-9 and integrin beta1 correlated with the HNSC migration. These results demonstrate

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that CD81 is key receptor in HNSC migration. The shRNA-mediated CD81 downregulation effectively reduced MMP-9 binding to the cell surface, which inhibited the migration of HNSC. Also, We performed Western-blot. That results show that CD81 stimulates the activity of ERK/MAPKinase in HNSC. Linked with this event, we observed an increase in CD81-associated phosphatidylinositol 3 - kinases activity and mTOR. Our results demonstrate that CD81 as a cell surface binding partner for MMP-9, regulating cell migration and adhesion via intergrin beta1 modulation of tetraspanin/integrin signaling complex. Also, it is well established that CD81 is Hub regulator in cell migration in HNSC cell migration.

Poster Board Number: T-1030

HUMAN CORD BLOOD DERIVED MULTIPOTENT STEM CELLS (CB-SC) TREATED WITH ALL TRANS RETINOIC ACID (ATRA) GIVE RISE TO DOPAMINE NEURONS

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Parkinson's disease (PD) results from the chronic degeneration of dopaminergic neurons. A replacement for these neurons has the potential to provide a clinical cure and/or lasting treatment for symptoms of the disease. Human cord blood-derived multipotent stem cells (CB-SCs) display embryonic stem cell characteristics, including multi-potential differentiation. To explore their therapeutic potential in PD, we examined whether CB-SCs could be induced to differentiate into dopamine neurons in the presence of all-trans retinoic acid (ATRA). Prior to treatment, CB-SCs expressed mRNA and protein for the key dopaminergic transcription factors Nurr1, Wnt1, and En1. Following treatment with 10μM ATRA for 12 days, CB-SCs displayed elongated neuronal-like morphologies. Immunocytochemistry revealed that 48±11% of ATRA-treated cells were positive for tyrosine hydroxylase (TH), and 36±9% of cells were positive for dopamine transporter (DAT). In contrast, control CB-SCs (culture medium only) expressed only background levels of TH and DAT. Finally, ATRA-treated CB-SCs challenged with potassium released increased levels of dopamine compared to control. These data demonstrate that ATRA induces differentiation of CB-SCs into dopaminergic neurons. This finding may lead to the development of an alternative approach to stem cell therapy for Parkinson's disease.

Poster Board Number: T-1031

VISUALIZING GLIOMA CELL ENGRAFTMENT TIME COURSE AND HUMAN NEURAL STEM CELL HOMING IN A XENOGRAFT MODEL USING PARAFFIN-EMBEDDED SERIAL SECTIONS.

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Studies of tumor cell engraftment and therapeutic efficacy in mouse xenograft glioma models commonly examine small tumor areas from a limited number of mice, despite evidence of considerable heterogeneity within tumors and between recipient mice. To be able to image entire tumors across many animals, we established procedures using conventional histological techniques by which engrafted tumors and neural stem cells (NSCs) could be analyzed in their entirety across multiple test animals using paraffin-sectioned material prepared by our local pathology core. Briefly, brains harboring engrafted tumors and associated NSCs were sectioned over the entire tumor. These sections were processed for chromogenic identification of tumor cells and NSCs, and slides spanning the entire tumor were automatically scanned at high resolution. A subsample of sections alternately stained for tumor and NSC markers were aligned, and segmented into tumor- and NSC-specific channels by computational color deconvolution. Algorithms developed for quantifying numbers of tumor- and NSC-occupied voxels were then used to quantify tumor volumes, numbers of NSCs, and predicted tumor coverage of NSC-delivered therapeutic agents. We used these procedures to analyze the engraftment time course of a tumor model originating from patient-derived tumor cells expanded in short-term culture, comparing histological results to those obtained by MR and bioluminescence imaging. We then examined the distributions of NSCs that intrinsically home to tumor sites following intracranial or intravascular administration. Low passage expanded patient-derived brain tumor cells (PBT017) were orthotopically implanted in brains of adult immunodeficient (SCID) mice. Engraftment kinetics were tracked by immunohistochemistry, and by MR and bioluminescence imaging, at time points between 1 and 9 weeks post-implantation. By all three measures tumor growth accelerated after the first 20-30 days, and tumor volume increased approximately 100-fold over the first 60 days. Ferumoxytol (iron oxide nanoparticle)-labeled cells from immortalized NSC line (HB1.F3.CD) were injected either by intracranial (i.c.) or intravascular (i.v.) routes, and homing to three week-engrafted tumors was visualized histologically four days post-NSC implantation by Prussian Blue staining. We found that NSC distributions varied with location of intracranial injection, and, for both i.c. and i.v. administrations, with tumor density and dispersion. Further progress in this area will involve sampling additional time points, examining tumor and brain heterogeneities related to these differences, and identifying signals to which the NSCs are responding.

Poster Board Number: T-1032

MODELING SPINAL MUSCULAR ATROPHY BY KNOCKING DOWN THE DISEASE-DETERMINING GENE IN HUMAN EMBRYONIC STEM CELLS

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Establishing human cell models of spinal muscular atrophy (SMA) to mimic motor neuron-specific phenotypes holds the key to understanding the pathogenesis of SMA. Here, we established human embryonic stem cell lines with the knockdown of the disease-determining gene, survival motor neuron (*SMN*). Neither neural induction nor spinal motor neuron specification is affected by knocking down of SMN. Notably, knocking down of SMN-full length (SMN-FL), but not SMN-delta 7 (lacking exon 7), results in impaired axonal outgrowth and subsequent neuronal degeneration. Moreover, all these phenotypes are specific to spinal motor neurons and can be rescued by restoring the expression of SMN-FL. Finally, in human motor neuron progenitors before the phenotype is observed, knockdown of SMN-FL leads to the increases in mitochondria oxidative stress and apoptosis, suggesting the involvement of these processes in the pathogenesis of SMA. Taken together, we demonstrate the successful establishment of a human SMA model, which exhibits disease gene isoform specificity, cell type specificity, and phenotype reversibility. This model provides a unique system to study the SMN gene function in human motor neurons and to explore how motor neurons are specifically degenerated in SMA.

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Poster Board Number: T-1033

SUSTAINED CLINICAL RECOVERY FOLLOWING INTRASPINAL TRANSPLANTATION OF HUMAN EMBRYONIC STEM CELL-DERIVED NEURAL PROGENITOR CELLS INTO A VIRAL MODEL OF MULTIPLE SCLEROSIS

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Transplantation of human neural progenitor cells (hNPCs) is a promising strategy for the treatment of demyelinating diseases. In this study, we surgically transplanted hNPCs differentiated from the human embryonic stem cell line WA09 into the spinal cords of mice persistently infected with the neurotropic JHM strain of mouse hepatitis virus (JMHV) in which demyelination and clinical disease were established. Clinical recovery was evident in >70% of JHMV-infected mice as early as day 8 post-transplant (p.t.) and was sustained up to 6 months. In contrast, transplantation of human fibroblasts did not result in any clinical improvement. At 7 days p.t., hNPCs were not detectable by immunohistochemistry within the CNS and natural killer cells, microglia, and neutrophils were aggregated at the implantation site, suggesting that rejection of hNPCs was mediated by the innate immune response. Mice were sacrificed at 21, 90, and 180 days p.t. and the effects on spinal cord pathology and neuroinflammation were evaluated. The hNPC treatment dramatically reduced both the spread of demyelination (as determined by Luxol fast blue staining) and neuroinflammation compared to control mice. Flow analysis and immunochemical staining revealed significantly less T cell infiltration and macrophage/microglia activation within the spinal cords of hNPC-transplanted mice compared to control mice. Co-culture of hNPCs with T cells purified from JHMV-infected mice resulted in dose-dependent dampening of T cell proliferation. Collectively, our results indicate that hNPCs are immunosuppressive and highlight that intraspinal instillation mutes the severity of neuroinflammation and demyelination, resulting in prolonged clinical recovery. These observations provide further support for hNPCs in treating inflammatory neurodegenerative diseases such as MS.

Poster Board Number: T-1034

CHANGE IN FUNCTIONAL PURINERGIC SIGNALING WITH COMMITMENT TO THE NEURONAL LINEAGE IN HUMAN FETAL CORTICAL NEURAL PRECURSOR CELLS

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Adenosine triphosphate (ATP) signals via P2X and P2Y purinergic receptors, and has important roles in neural stem cell (NSC) proliferation and differentiation in rodents. During neuronal differentiation, ATP release decreases and purinergic receptor expression is downregulated, leading us to hypothesise purinergic signaling may have significant roles in human neurodevelopment. This study used calcium (Ca^{2+}) imaging of cultured human neural precursor cells (hNPCs) in adherent neurosphere monolayers from human fetal cerebrum. hNPCs showed a rapid, substantial increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) in response to ATP, adenosine diphosphate (ADP), 2-methylthioATP (2-MeSATP), uridine triphosphate (UTP) and uridine diphosphate (UDP). The response was predominantly from thapsigargin-sensitive intracellular stores. ATP-stimulated cells showed 3 distinct responses (single, double or multiple $[Ca^{2+}]_i$ rises); the latter an oscillatory response which propagated between adjacent cells. Post Ca^{2+}-imaging immunostaining revealed ADP and ATP evoked oscillations in immature nestin$^+$/s100β$^+$/βIII-tubulin$^+$ hNPCs whereas nestin$^-$/s100β$^-$/βIII-tubulin$^+$ immature neurons never oscillated and showed lower responses. Application of the P2Y1-receptor antagonist (MRS2179) reduced the ADP response and oscillations. The P2X1/P2X3 agonist (α,β-methylene ATP) and the P2X7 agonist (BzATP) showed low to moderate responses. The delayed BzATP response was characteristic of P2X7 receptor pore opening, and two specific P2X7 receptor antagonists significantly reduced both responses, confirming the specificity of the response to the P2X7 receptor. Immunostaining confirmed the expression of P2Y1, P2X1, P2X3 and P2X7 receptors. This is the first identification of functional P2Y and P2X receptors in hNPCs and immature neurons, and provides unique insights into the developmental changes which occur with commitment to the neuronal lineage.

Poster Board Number: T-1035

BAC-BASED ASSESSMENT OF PROTEIN-PROTEIN INTERACTIONS IN HUMAN PLURIPOTENT STEM CELL-DERIVED NEURAL STEM CELLS AND THEIR NEURONAL PROGENY

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Protein interaction studies represent a powerful tool to study cell signaling cascades, cell-cell interactions as well as principles of signal transduction mechanisms. Up to today, the majority of human interactome studies were conducted based on overexpression paradigms in tumor cell lines. Commonly encountered problems in this context are unspecific interactions due to supra-physiological protein expression levels, the use of transformed cells and a non-tissue specific proteome. Ideally, protein-protein interaction data should be generated in the appropriate tissue-specific somatic cell expressing the protein of interest at endogenous expression levels. Here we used pluripotent stem cell-derived neural stem cells (lt-NES®; Koch et al., PNAS 106:3225-30, 2009) as a somatic stem cell population, which exhibits extensive self-renewal, clonogenicity and stable neurogenesis. The introduction of GFP-tagged proteins via bacterial artificial chromosomes (BAC; Poser et al., Nat. Meth. 5:409-15, 2008) permitted the derivation of large numbers of polyclonal cell populations (pools) with faithful protein expression in more than 90% of the cells, average integration rates of one locus per genome as well as correct size and compartmentalization of the tagged variants as detected by Western Blot analysis and high-resolution live cell imaging. Using this technique, we generated multiple cell lines harboring tagged proteins including PCNA, JARID1C, AURKA, CDK2AP1, RBPJ, RUVBL2, the Methyl CpG Binding Protein 2 (MECP2) involved in the pathogenesis of Rett syndrome and the Alzheimer's disease-associated proteins Nicastrin (NCSTN) and Valosin-containing protein (VCP). Using

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protein-protein interaction studies with a label-free, quantitative affinity purification-mass spectrometry approach (Hubner et al., J Cell Biol 189:739-54, 2010) we identified several known complexes as well as novel interaction candidates of these proteins. The system is sufficiently sensitive to report changes in protein expression levels and compartmentalization during lt-NES® cell differentiation and after exposure to extrinsic factors such as inhibitors of proliferation and modulators of cell signaling. Furthermore, we were able to transfer this technique to iPS cell-derived lt-NES® cells from patients with Machado-Joseph disease (UbC, VCP) and Alzheimer's disease (VCP, NCSTN). Our data suggest that protein tagging in PSC-derived lt-NES® cells and their neuronal progeny represents an efficient approach for studying protein-protein interactions in human neural cells both in normal and neurodegenerative contexts.

Poster Board Number: T-1036

GABAERGIC NEURONS DURING EARLY HUMAN BRAIN DEVELOPMENT

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Human forebrain GABAergic neurons comprise of those carrying various biochemical and transmitter phenotypes, including DARPP32 (Dopamine and Adenosine 3',5' Monophosphate Regulated Phosphoprotein), somatostatin (SOM), parvalbumin (PV), and calretinin (CR). They play versatile role in brain functions. It is not clear if these subtypes of GABA neurons are derived from neural progenitors in distinct regions during human development. At 7-8 gestational weeks, GABAergic neurons were observed in the ventral telencephalon at the lateral ganglionic eminence (LGE). These GABAergic projection neurons co-express Islet1 and DARPP32 but PV and CR. Interestingly, we also observed a group of GABA neurons at the corticostriatal border that express the cortical transcription factors Pax6. A small part of these Pax6 immunoreactive neurons also express DARPP32. By 8 gestational week, we began to observe GABA neurons in MGE, LGE, and they co-express NKX2.1 . Later, we found a goup of GABA neurons migrate to cortical plate coexpressing NKX2.1 but Pax6. It suggested that altuough most part of the GABAergic originate from ventral telencephalon, there is a small part of GABAergic neurons born from dorsal origin.

Poster Board Number: T-1037

HUMAN NEUROEPITHELIAL STEM (NES) CELLS -A STANDARDISED SYSTEM FOR COMPARISON STUDIES

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Robust and standardised cultures of human stem cells provide a platform for comparison studies. Human NeuroEpithelial Stem (NES) cells can be derived from embryonic stem (ES) cells and induced pluripotent stem (iPS) cells. Interestingly, NES cells can also be captured directly from the foetal human brain demonstrating their existent during development. NES cells of different origins display comparable characteristics including long-term proliferation in culture without loosing their ability to differentiate to functional neurons and glia. NES cells have a hindbrain identity and a characteristic transcription factor profile. The consistency between NES cells of various origins make them an interesting tool for comparative analyses of neural cells from control and diseased genetic backgrounds. NES cells are expanded in EGF and FGF2 supplemented media over 100 passages maintaining a stable karyotype and a neuronal differentiation potential creating up to 90% neurons upon growth factor withdrawal. NES cells grow with an organised rosette-like morphology expressing neuroepithelial and neural stem cell markers including Sox1, Sox2, Nestin, Dach1 and PLZF. NES cell cultures are stabilised in a hindbrain positional identity during the first 10-15 passages, expressing Gbx2, Krox20, Irx3, and Nkx6.1.

Poster Board Number: T-1039

IN VITRO AND *IN VIVO* DIFFERENTIATION OF HUMAN FETAL NEURAL PROGENITORS.

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Introduction: Neural stem cells (NSC) reside within the central nervous system (CNS), and have been propagated as neurospheres (NS) in culture. They are intended for therapy particularly in neurodegenerative diseases which are of increasing prevalence due to aging populations in developed countries. NSC have been isolated from several parts of the developing human fetal brain and here, we attempt to profile these cells for CD133 expression as well as investigate both their in vitro and *in vivo* differentiation potential. Methods: Fetal brain tissues were collected from medically-indicated pregnancy terminations between 13-15 weeks gestation, with informed consent. Tissues from second trimester subventricular zone, hippocampus, anterior and posterior cerebra, thalamus, cerebellum, brain stem and spinal cord were minced and dissociated enzymatically (n=4). Serum-free NS culture was performed over 2-4 weeks, and C133 profiling performed at every passage. Neurospheres at passage 1 were subcultured and placed into differentiating medium to assess in vitro dopaminergic differentiation. Concurrently, NS were also transduced green and 15X103 cells were injected into CD1 pup at E14 to determine their *in vivo* differentiation. Immunocytochemistry for MAP2ab, tyrosine hydroxylase (TH) on the cultured cells and nestin, BIII-tubulin, glial fibrillary acidic protein (GFAP) on the animal brain sections were performed to determine final identity of differentiated cells. Results: We isolated NS from cells derived from the different regions of the human fetal brain. Expression of CD133 at point of harvest ranged from 0% and 12.5% which was observed in the spinal cord. At passage 1, expression of CD133 then ranged from 0% to 30.9% which was observed in the cerebrum. Following that, a sharp decrease in expression of CD133 was observed which continued through passage 3.In vitro, dopaminergic differentiation can be observed from all cultures derived from all regions of the fetal brain and proportion of MAP2ab+TH+ cells ranged from 2.4±1.2% in cultures from anterior cerebrum to 34.4±8.8% in cultures differentiated from brain stem-derived NSC. From the *in vivo* data, successful engraftment of labelled human neural progenitors was observed in 1 out of the 7 pups injected. Engraftment was observed 4 weeks post injection and stained brain sections demonstrated the differentiation of the human neural stem/ progenitors into GFAP+ glial cells and the absence of undifferentiated nestin+ human neural stem/ progenitors. Conclusion: If CD133 is a bona fide neural stem/ progenitor marker, our data suggests the greatest proportion of neural stem/ progenitors at passage 1 of NS cultures. Our differentiation studies demonstrated the successful differentiation of these human fetal neural stem/ progenitors into dopaminergic neurons in vitro and glial cells *in vivo*.

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ACTIVATION OF RHO-A PROMOTES NEURONAL DIFFERENTIATION FROM MICRO RNA REGULATED HUMAN BRAIN NEURAL STEM CELLS

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Introduction: Commitment of neural stem cells to differentiate into functional neuronal cells is an essential step in pursuing cell therapy for neurological disorders. Neuronal differentiation (ND) involves many endogenous and exogenous steps such as cell-cycles, intracellular signals, cell structural proteins and cell shape alterations. The previously reported results suggest that both miRNA let7s and RhoA expressions play critical roles on ND. Lower levels of RhoA and Rho GTPases mediate cadherin adhesion molecules to up-regulate stem cell proliferation and expansion, whereas high levels of RhoA expression affect ND. Data from *in vitro* studies have shown that ND can be directly derived from embryonic stem cell cultures after manipulation of miRNA let7s expression. However, there are a few reports addressing the mechanism of endogenous signals regulating the morphological and genetic changes of human brain neural stem cell (HBSC) during ND. Furthermore, how miRNA let7 and RhoA initiate and govern final ND from HBSC also remains unclear. The purpose of this study was to observe the ND from HBSC culture treatment by the miRNA let7/RhoA expression enhancers such as β-1 integrin and EphrinA1 respectively. Results: To further understanding, the molecular mechanism of ND from HBSC (nestin^{+}/CD133^{+}) gene and protein expression profiles of HBSC treated with or without β-1 integrin and EphrinA1 were analyzed using cDNA microarray representing 11000 mRNAs. The significant alterations in RhoA and RhoA-Rho kinase (RAK) expression levels were also confirmed using RT-PCR, Northern blotting and protein assays. OCT4, SOX2 and Nonog were expressed differently between the cell treated with and without β-1 integrin and EphrinA1. Compared with non-treated cultures, both RhoA and RAK genes were up-regulated respectively (4 to 7 times respectively) after β-1 integrin and EphrinA1 treatment. We also found that both OCT4 and SOX2 expressions increase following elevated Rho/RAK expression. Neuronal specific protein markers (such as Hu, Neu-N, NF-L, N-cadherin, β-III Tubulin and MAP-2) were positively detected in the up-regulated RhoA cultures. In contrast, suppression of the RhoA and RAK mRNA expression with anti-RhoA/RAK antisenses significantly suppresses the ND. These data suggest that the RhoA/RAK signaling pathway is critical for ND differentiation. However, to confirm that the differentiated cells are the functional neuronal cells will require further electronic physiological functional test. Material Methods: Cell culture Protocols for human fetal brain cell isolation, primary cell culture maintenance and differentiation, have been proved by IHC of WSU and Guangzhou 34air force hospital. miRNA expression profiling Total RNA samples were extracted from untreated NSCs, cells treated with *Let7* oligonucleotide or β-1 integrin for 5 days, and cells cultured within serum-free medium for 7 days. Detection of NSC and neuronal mRNA and protein expression from cell cultures The transcription levels of nestin and neuronal specific protein makers were analyzed by reverse transcriptase (RT)-PCR and immunocytochemistry stains.

Poster Board Number: T-1041

MRI TRACKING OF FERUMOXYTOL-LABELED HUMAN NEURAL STEM CELLS: IMPLICATIONS FOR CLINICAL USE

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Stem cells are under investigation as therapeutic agents for multiple types of cancers, including gliomas. A first-in-human study of neural stem cell (NSC)-mediated treatment of recurrent glioma is currently in Phase I clinical trial. Monitoring the movements and fates of therapeutic stem cells in patients is critically needed in cell-mediated therapies, however such methodology has not been developed yet. A promising method for monitoring NSCs involves loading the cells with iron nanoparticles to enable subsequent tracking of their migration and tumor distribution over time with magnetic resonance imaging (MRI), which would allow for optimization of treatment regimens and strategies. In this study we evaluated the pre-clinical safety of labeling NSCs using a recently developed method for iron labeling of stem cells using the combination of heparin (H), protamine (P) and ferumoxytol (F) (HPF) and optimized MRI monitoring of HPF-labeled NSCs *in vivo*. HPF labeling of NSCs with nanocomplexes did not affect cell viability, growth kinetics or tumor tropism when tested *in vitro* and *in vivo*. MRI revealed dynamic *in viv*o NSC tumor tropism at multiple time-points after intracranial or intravenous injection of NSCs into glioma-bearing mice that correlated with data obtained by histological examination. Intracranial administration of HPF-labeled NSCs did not cause clinical or behavioral changes in mice, no accumulation of iron was observed in the liver and spleen, and no local neuronal or systemic toxicities were detected. This study is a significant contribution to development of using HPF-labeled NSCs in clinical settings for treatment of brain tumors and other therapeutic indications.

Poster Board Number: T-1042

BIDIRECTIONAL CONTROL OF THE COMPETENCE OF MOUSE AND HUMAN NEURAL STEM CELLS

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Central nervous system (CNS) injury and disease have gained new prominence in modern medicine. According to the recent progress in stem cell biology, human neural cells can be readily obtained from induced pluripotent stem cells (iPSCs) and induced neuronal cells (iNCs). These are expected as innovative sources of biological materials for transplantation therapy and drug screening. However, the specific and efficient induction of homogeneous target cell populations remains difficult due to following reasons: (1) the neuropotency of neural stem cells (NSCs) often depends on epigenetic status of iPSC origin; (2) the mechanisms of NSC cytogenesis including spatio-temporal regulation of the differentiation potential is quite complex; and (3) iNCs are not expandable. Here, we figured out a solution to this problem by elucidation of molecular machinery of a novel dynamic regulatory program in NSC cytogenesis properties, namely the "competence change". Although we hypothesized the "competence change" mediated by Coup-tfl and II (Coup-tfs) as the machinery of the temporal changes in responsiveness of NSCs to gliogenic cytokines in Nature Neuroscience in 2008, molecular mechanisms underlying it have been completely unknown. NSCs diminish their neuropotency and acquire gliogenic

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competence during development, therefore, efficient induction of neurons is difficult from NSCs after midgestation. Here, we identify the effector for the Coup-tfs-mediated neurogenic-to-gliogenic transition of NSC competence. Its overexpression not only impaired the acquisition of gliogenic competence by developing NSCs, but also restored neuropotency even in stage-progressed gliogenic NSCs, without aberrant alterations in the epigenetic status of the promoter of the gene for Gfap, a glial marker. The restoration of neuropotency by inhibition of the effector was also observed in human fetal brain-derived NSCs. In the field of stem cell research, the differentiation processes has been likely to be main focus toward the control of cytogenesis by stem cells for their application. However, the control of differentiation processes is often dependent on their contexts. Case in point: it is difficult to induce many neurons from gliogenic NSCs at late developmental stage because they already diminish their neuropotency. Therefore, the competence regulation may open the way to maintain and recover the neurogenic potential of NSCs.

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ELEVATION OF REACTIVE OXYGEN SPECIES STIMULATES THE MIGRATION OF IRRADIATED MOUSE AND HUMAN NEURAL PROGENITOR CELLS

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Neural progenitor cells (NPCs) migration is an essential process for brain development. Whereas in vivo studies report abnormal neuronal migration in cerebral cortex in mice prenatally exposed to ionizing radiation, little is known on the consequences of irradiation on NPCs migration. In this study, we showed that, twenty-four hours after irradiation, wild type cortical mouse NPCs (mNPCs), as well as human NPCs (hNPCs), displayed an enhanced migration velocity associated with an elevated level of Reactive Oxygen Species (ROS) compared to non-irradiated controls. Similar increase of migration was observed in non-irradiated Rad54 deficient mNPCs, cells that spontaneously exhibit a high endogenous ROS level compared to wild type mNPCs. In these different cellular models, the antioxidant NAC treatment restored migration capacities similar to that observed in non-irradiated wild type NPCs. These data suggest that the oxidative stress generated after a γ-irradiation or endogenously found in Rad54 deficient NPCs, is able to stimulate migration of NPCs cells. Using NPCs from WT/Fucci and Rad54/Fucci mice, that permit us to visualize nuclei in S/G2 or in G1 phase respectively by green or red fluorescence, we found that the increase of migration velocities reported in irradiated NPCs or in Rad54 mNPCs was not associated with a modification of cell cycle progression. Lastly, we evaluated the effect of γ-irradiation on NPCs migration on organotypic brain slice and after intracranial transplantation of GFP NPCs. These *in vivo* experiments definitively showed that oxidative stress resulting from γ-irradiation enhanced migration of both mouse and human embryonic neural progenitor cells.

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CELL LINEAGE TRACING OF SOX2 EXPRESSING CELLS IN THE ADULT MOUSE BRAIN

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Sox2 encodes a transcription factor which plays important roles in regulating neural development. It is also believed that Sox2 is one of the neural stem cell markers in the adult brain. There are two major neurogenesis zones in the mouse brain, which include subgranular zone of hippocampal dentate gyrus and subventricular zone of lateral ventricle. Here we take a genetic approach to label Sox2 positive cells in the adult mice brain by crossing a Sox2-CreERT2 transgenic mouse with an inducible organelle specific dual fluorescent protein reporter mouse (R26RGR). The dual fluorescent proteins are encoded by an inducible transgenic construct (H2B-EGFP-2A-mCherry-GPI) placed in the Rosa26 locus. The H2B-EGFP encodes a histone protein fusion with an enhance green fluorescent protein which allows us to observe chromatin in the nucleus, providing cell cycle information including mitosis, while mCherry-GPI encodes red fluorescent membrane anchored protein depicting cellular locations and morphologies. Under the induction of tamoxifen at the adult stage, we can label Sox2 positive cells, putative neural stem cells and trace their descendents *in vivo*.

Poster Board Number: T-1045

EXPRESSION PATTERN OF GFP DRIVEN BY FGF1 PROMOTER IN F1B-GFP TRANSGENIC MICE

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Fibroblast growth factor 1 (FGF1) is expressed predominantly in the neural tissues, such as brain and retina. In the normal mouse brain, FGF1 expression can be detected in the sensory and motor nuclei in the brain stem, cerebellum and substantia nigra. FGF1 has been shown to maintain self-renewal and multipotent capacities of neural stem/progenitor cells (NSPCs) in vitro. Previous studies showed that 571-bp (-540 to +31) sequence of the brain-specific FGF1 promoter (F1B) is sufficient to drive the expression of a heterologous luciferase reporter in cultured cells. Also, F1B, as the major transcript of FGF1 gene, is expressed in brain areas that are known to be abundant for NSPCs *in vivo*. In order to identify the function of F1B in brain, we generated F1B-GFP transgenic mice, which contain 571-bp F1B promoter sequence and fused to green fluorescent protein (GFP) as a reporter gene. Southern blots showed higher intensity signals in #115G F1B-GFP mice than other founders, indicating the #115G contains a higher copy number of the transgene. We further extracted total RNAs from brains of 8-week old mice. Using reverse transcription and PCR techniques, we found that GFP was expressed in whole brain. Immunohistochemistry (IHC) was conducted to label the F1B-GFP+ cells in mouse brains. The IHC results from tissue sections of 12-week old mouse brains showed F1B-GFP+ cells expressed in two population of cells. One population of F1B-GFP+ cells were ependymal cells which resided in ventricle system, including lateral ventricles (LV), dorsal third ventricle (D3V), third ventricle (3V), aqueduct (Aq) and central canal (CC). The second population of F1B-GFP+ cells was neural cells. We found that a part of F1B-GFP+ cells can express the dopaminergic neuron marker, tyrosine hydroxylase. In addition, we showed that F1B-Cre

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transgenic mice, mated with Rosa26 transgenic mice to detect β-galactosidase activity, also exhibited the same expression pattern as F1B-GFP mice. Taken together, we successfully used mouse as an animal model to express human gene promoter, F1B, and the F1B-GFP+ cells are ependymal and neural cells. This F1B-GFP transgenic mouse might provide a novel tool to understand FGF1 function in brain development and in related diseases.

Poster Board Number: T-1046

ENHANCED IL-1BETA PRODUCTION IN RESPONSE TO THE ACTIVATION OF HIPPOCAMPAL GLIAL CELLS IMPAIRS NEUROGENESIS IN AGED MICE

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A variety of mechanisms that contribute to the accumulation of age-related damage and the resulting brain dysfunction have been identified. Recently, decreased neurogenesis in the hippocampus has been recognized as one of the mechanisms of age-related brain dysfunction. However, the molecular mechanism of decreased neurogenesis with aging is still unclear. In the present study, we investigated whether aging decreases neurogenesis accompanied by the activation of microglia and astrocytes, which increases the expression of IL-1beta in the hippocampus, and whether *in vitro* treatment with IL-1beta in neural stem cells directly impairs neurogenesis. Ionized calcium-binding adaptor molecule 1 (Iba1)-positive microglia and glial fibrillary acidic protein (GFAP)-positive astrocytes were increased in the dentate gyrus of the hippocampus of 28-month-old mice. Furthermore, the mRNA level of IL-1beta was significantly increased without related histone modifications. Moreover, a significant increase in lysine 9 on histone H3 (H3K9) trimethylation at the promoter of NeuroD (a neural progenitor cell marker) was observed in the hippocampus of aged mice. *In vitro* treatment with IL-1beta in neural stem cells prepared from whole brain of E14.5 mice significantly increased H3K9 trimethylation at the NeuroD promoter. These findings suggest that aging may decrease hippocampal neurogenesis via epigenetic modifications accompanied by the activation of microglia and astrocytes with the increased expression of IL-1beta in the hippocampus.

Poster Board Number: T-1047

INDUCED NEURONAL CELLS FROM AGING MOUSE FIBROBLASTS

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Defined sets of transcription factors have recently been used to convert mouse and human fibroblasts into cells with neuronal characteristics. These induced neuronal cells (iN cells) show all hallmarks of normal mature neurons. However, it is still unknown if iN cells can be generated from aged cells and whether there are any changes in reprogramming efficiency or function of aging iN cells. Aging is associated with declined cognitive function, changes in neural plasticity and reduced neurogenesis. On the other hand it is difficult to study intrinsic neuronal properties of aged mammals. It is virtually impossible to culture aged neurons; it is hard to isolate them in high purity and challenging to assess their electrophysiological properties. It would therefore be desirable to generate aged iN cells in order to study age related changes in neurons. Here we describe the generation of iN cells from aging, postnatal to aged 25 month old, mouse fibroblasts. Tail derived fibroblast cultures were established from postnatal, 4 day old and 3, 10, 18 and 25 month old mice. Fibroblasts were infected with doxycycline-inducible lentiviral vectors carrying Brn2, Ascl1 and Myt1l and were assayed for iN cell generation and function at 2-3 weeks after transgene induction. iN cells were generated at similar efficiencies from adult to aged fibroblast. The efficiency was however lower as compared to those generated from embryonic fibroblasts. Aging iN cells were positive for pan neuronal markers Tuj1 and Map2. They displayed mature neuronal morphologies without any change in complexity between age groups as assessed by neurite tracing. To probe the functional properties of these iN cells, we next performed current-clamp experiments. iN cells derived from different ages had similar resting membrane potential, input resistance, and capacitance. Upon current injection, iN cells from different ages generated single or multiple action potentials with similar efficiency. Furthermore, the action potential threshold or amplitude did not change significantly across all ages tested. Finally, we performed voltage-clamp experiments to measure sodium and potassium conductance of the iN cells and found no difference between the ages. We here show that iN cells can be generated from aged mouse fibroblast and suggest that these findings may be useful for studying neuronal aging and to model age related changes in neurogenesis.

Poster Board Number: T-1048

EFFICIENT GENERATION AND DEVELOPMENTAL ANALYSIS OF BASAL FOREBRAIN CHOLINERGIC NEURONS FROM MOUSE EMBRYONIC STEM CELLS

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The differentiation mechanisms of basal forebrain cholinergic neurons (BFCN) in early neural development are of special interest, because dysfunctions of BFCN have been implicated to be involved in cognitive impairments including Alzheimer's disease. However, little is known about the underlying mechanism of the development of early-born BFCN. For the analysis of the development of BFCN, we investigated the roles of Lhx8, which is expressed in medial ganglionic eminence (MGE) and is known to positively regulate cholinergic differentiation, using an *in vitro* embryonic stem (ES) cell differentiation system to induce BFCN. To obtain BFCN from mouse ES cells, we first differentiated ES cells as serum-free, floating culture of embryoid body-like aggregates (SFEB) with Dkk1. Dkk1 not only increased the number of Sox1-GFP+ cells in SFEB but also up-regulated the expression of forebrain marker, Foxg1, and a MGE marker, Nkx2.1. Thus, we then dissociated Dkk1-treated SFEBs into single cells and cultured them in suspension with FGF-2 to form neurospheres and to differentiate them into BFCN. Immunocytochemical analysis showed efficient differentiation into βIII-tubulin/choline acetyltransferase (ChAT) double positive BFCN. Also, NGF increased the number of ChAT-positive BFCN, and the expression of chat by 40-fold, suggesting that our *in vitro* system is useful for generating ChAT-positive BFCN from mouse ES cells. Next, we carried out lentivirus-mediated knockdown of Lhx8 and Lhx6 by short-hairpin RNAs (shRNAs) during neurosphere formation in our *in vitro* system. Consistent with the previous reports showing a significant decrease in the number of BFCN in Lhx8-null mice, in our system the expression of ChAT was decreased by the knockdown of Lhx8 under differentiation conditions. These results suggest that the dif-

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ferentiation potential into BFCN is positively regulated by Lhx8. To identify the downstream transcription factors of Lhx8, we collected GFP-positive cells in neurospheres by fluorescence-activated cell sorting and performed microarray analysis comparing cells infected with Lhx8-shRNA to those infected with a control virus. As a result, we identified 963 probes (819 genes) whose expression levels changed more than two-folds by the knockdown of Lhx8. Among these, we identified 54 transcription factors by gene ontology analysis. Also, Lhx8 is expressed at 114-fold higher level in neural progenitors prepared from forebrain at E11.5 compared with those at E14.5; therefore we picked up 31 genes highly expressed in E11.5 neural progenitors. Future studies should include the identification of downstream transcription factors of Lhx8 by investigating the roles of these genes in the development of BFCN.

Poster Board Number: T-1049

JARID 2 REGULATION OF MURINE POSTNATAL SUBEPENDYMAL ZONE NEUROGENESIS

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The murine subependymal zone (SEZ) contains stem cells and produces daily tens of thousands of neurons.. However, the mechanisms of SEZ neural stem/progenitor cell (NSPC) maintenance, differentiation and cell-fate specification are still not clear. To understand the role of epigenetic factors in this process, we are investigating whether Jarid2/Jumonji is necessary for regulating self-renewal and multipotency of SEZ NSPCs. Jarid2 interacts with polycomb repressive complex 2 (PRC2) in embryonic stem cells to regulate the balance between self-renewal and differentiation by modulating pluripotency genes. The expression level of Jarid2 and PRC2 core components such as Ezh2, Eed and Suz12 in postnatal mouse SEZ NSPCs was previously unknown; therefore, we have now shown their expression *in vivo* and *in vitro* as neurospheres with qRT-PCR and Western blot. Using lentiviral-delivered shRNAs in the SEZ neurospheres, we found Jarid2 knockdown increased the number of neurospheres but decreased their diameter and the number of differentiated cells. Eed knockdown in neurospheres, however, did not show any significant difference in their self-renewal and multipotency, compared to the scrambled control. Our data suggest that Jarid2 is necessary for SEZ NSPC differentiation. In addition, different neurosphere phenotypes after knocking down Jarid2 and Eed imply Jarid2 may have as yet unknown PRC2-independent roles to govern the postnatal SEZ NSPCs. Understanding the role of epigenetic factors in the context of NSPC self-renewal and differentiation has important implication for the development of neural stem cell-based therapies.

Poster Board Number: T-1050

INVESTIGATION OF THE OPTIMAL TRANSPLANTATION SITE OF FETAL NEURAL STEM/PROGENITOR CELLS FOR SPINAL CORD INJURY IN ADULT MICE.

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There were many reports that transplantation of neural stem/progenitor cells (NS/PCs) promoted functional recovery in several animal models of spinal cord injury (SCI) and we have recently reported intra-lesional transplantation of NS/PCs was the most effective compared to the other procedures; intra-thecal and intra-venous transplantation. However, there have been no report to determine where exactly the best transplantation site is, because previous researchers grafted NS/PCs into various sites of injured spinal cord; epicenter, rostral and caudal sites and so on. The purpose of this study is to determine the optimal transplantation site of NS/PCs for SCI in adult mice. Adult female C57BL/6J mice received 60 kdyn contusion injuries using the Infinite Horizon Impactor after laminectomy at the Th10 level. 9 days after SCI, NS/PCs derived from the fetal transgenic mice, which ubiquitously expressed Venus and luciferase, were transplanted into the injured spinal cord at different sites: the lesion epicenter (E-group), and 1 mm rostral and caudal sites from the rim of the lesion (RC-group). Survival of NS/PCs was quantitatively evaluated using *in vivo* bioluminescence imaging. Motor function of hindlimbs was assessed by Basso Mouse Scale (BMS) for 6 weeks after SCI. Thereafter, the animals were sacrificed and immunohistochemical staining was processed for histological analysis. There was no difference in BMS between the E-group and RC-group. The final photon counts of the grafted NS/PCs converged within a narrow range in the both groups, independent with the initial photon counts. Grafted NS/PCs differentiated into neurons, astrocytes and oligodendrocytes. In the RC-group, grafted NS/PCs differentiated into more neurons and less astrocytes compared to the E-group. There was no significant difference in the distribution of the transplanted cells, positive areas of Neurofilament 200kDa (NF-H) and platelet endothelial cell adhesion molecule-1 (PECAM-1) between the both groups. The final photon counts were convergent and independent with the initial photon counts, suggesting that the survival of grafted NS/PCs was fairly constant if more than a certain number of the cells were transplanted. Although the differentiation pattern of the grafted NS/PCs were different between the two groups, there was no significant difference in the functional recovery and the final survival of NS/PCs. Therefore, it is estimated that there would be no difference in microenvironment between the epicenter, rostral and caudal sites at the sub-acute phase of SCI. Collectively, we conclude that the optimal transplantation application is to transplant more than a certain number of NS/PCs into the epicenter of injured spinal cord at the sub-acute phase, considering the additional damage of the intact spinal at the site to the lesion epicenter by the injection procedure.

Poster Board Number: T-1051

MODULATION OF NEURAL STEM CELL PROLIFERATION BY THE EXOGENOUS GENE EXPRESSION ENHANCED BRAIN RECOVERY IN A RAT MODEL OF ISCHEMIC STROKE

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Brain repair after ischemic injury largely depends upon neurogenesis of endogenous neural stem cells, suggesting that enhancement of neural stem cell proliferation would be a strategy for treating many brain diseases. In a series of experiments, we tested if several secreted proteins potentially modify brain microenvironment in a favorable way for neurogenesis and brain repair. For this, we overexpressed the candidate proteins in the ischemic penumbral area of Sprague-Dawley rats 3 days before transient middle cerebral artery occlusion (MCAO). In another set of experiment, the secreted factors were delivered into the brain 1 week after MCAO which corresponded to the subacute stage of stroke. Behavioral recovery was assessed for 5 weeks with a battery of behavioral tests including modified neurologic severity score (mNSS), accelerated rotarod and foot fault tests. We found that, among the factors, SDF1 demon-

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strated a significant improvement of neurologic deficits resulting from ischemic brain injury. BrdU-labeling experiments demonstrated newborn (BrdU+) cells in the SVZ and striatum were dramatically increased by the exogenous SDF1 and most of the BrdU+ cells were co-stained with doublecortin and PSA-NCAM. Convincingly, the number of BrdU+ cells was highly correlated with the results of behavioral tests. We also found that the number of newly formed neurons and glial cells in the peri-infarct area was increased by the overexpressed SDF1. In summary, we observed the therapeutic efficacy of SDF1 regardless of delivery time post-stroke, indicating the therapeutic potential of this protein in treating stroke patients of various stages of stroke. (This research was supported by *Stem Cell Research Program (2010-0020407) funded by the Ministry of Education, Science and Technology of Republic of Korea*).

Poster Board Number: T-1052

PERLECAN IS NECESSARY FOR THE MAINTENANCE OF CD133 EXPRESSING NEURAL STEM CELLS IN THE ADULT MOUSE SUBVENTRICULAR ZONE.

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In the subventricular zone (SVZ) of the adult brain, GFAP+ neural stem cells (NSCs) are tightly associated with fractones and blood vessels basement membranes. Perlecan, a major heparan sulfate proteoglycan (HSPG) is present in the basement membranes of the SVZ blood vessels and in fractones. However, the role of perlecan in the neurogenic niche has remained largely unknown. Perlecan-null mice die at birth because of premature cartilage development. To restore cartilage abnormalities, we created lethality-rescued mice, which expressed recombinant perlecan in cartilage, using a cartilage-specific Col2a1 promoter/enhancer. In this study, using this mouse model deficient in perlecan in the adult brain, we assessed the content of extracellular matrix in the neurogenic niche and investigated the behavior of NSCs in the absence of perlecan. We observed no obvious change in the constitution and integrity of the blood vessel basement membranes and in the fractones of the SVZ despite the absence of perlecan. To discriminate GFAP+ NSCs from GFAP+ SVZ astrocytes, we used the stem cell marker CD133. We observed a decrease in the number of CD133+GFAP+ NSCs in the SVZ of perlecan null mice. Finally, the number of new neuroblasts (expressing the immature neuronal marker doublecortin) in the SVZ and the integration of new neurons (expressing the mature neuronal marker NeuN) in the olfactory bulb were also decreased in perlecan null mice. These results suggest that perlecan is necessary for the maintenance of CD133+GFAP+ NSCs population and for neurogenesis. This study brings the first evidence of a key role of perlecan in the adult neural stem cell niche.

Poster Board Number: T-1053

HISTAMINE PROMOTES NEURONAL DIFFERENTIATION OF CULTURED RAT MIDBRAIN NEURAL PRECURSOR CELLS BUT DIMINISHES DOPAMINE NEURON NUMBERS IN VITRO AND *IN VIVO*

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Histamine (HA) is a neuroactive molecule that is involved in the regulation of diverse physiological functions in adult organisms, such as wakefulness and sleep, motor activity, as well as thermic and endocrine modulation. During central nervous system (CNS) development, histamine (HA) is one of the first neurotransmitters to appear, presenting higher concentrations than those observed in the adult brain. Particularly, in the region of ventral midbrain (VM), HA increases its concentration up to 5-fold at embryonic days 14-16 and then its levels are decreased close to birth, reaching the low concentrations that are present in the adult brain. Interestingly, neuronal differentiation in the VM correlates temporally with the increase in the concentration of HA in this region. The role of HA during brain development has been elusive. In this work, we aimed to study the effects of HA on neural precursor cells (NPC) of rat VM in vitro and *in vivo*. We analyzed the effect of several HA concentrations on proliferation, differentiation and cell death of cultured VM NPC. We found that VM cells express HA receptors, and that HA was able to significantly increase neuronal differentiation from 21% to 37%. Pharmacological assays revealed that this HA-induced neuronal differentiation is due to activation of H2 receptors. We observed that HA increased 2-fold apoptotic cell death compared to control conditions, evaluated by TUNEL assay. An interesting finding was that the proportion of dopaminergic neurons was selectively and significantly decreased after HA treatment of VM NPC. We also evaluated the role of HA *in vivo* through intrauterine injections in the developing brain, and found that HA administration decreased the number of dopaminergic neurons in the VM, without affecting gabaergic neurons. Taken together, these results suggest that HA might be acting as a transient signal that modulates neurogenesis and dopamine neuron differentiation/survival in the midbrain during CNS development.

Poster Board Number: T-1054

REGULATION OF ADULT MOUSE NEURAL STEM CELLS BY TLR9 MEDIATED SIGNALING.

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Adult tissue-specific stem cells possess the ability to self-renew and generate functionally differentiated cells that replenish lost cells throughout an organism's lifetime. Stem cell functions are controlled by extracellular cues from the niche and by intrinsic genetic programs within the cells. Recent studies have shown that the adult mammalian brain retains neural stem cells (NSCs) in the subventricular zone (SVZ) of the lateral ventricle and subgranular zone (SGZ) of the hippocampal dentate gyrus. NSCs generate three major cell types in the central nervous system (CNS), i.e., neurons, astrocytes and oligodendrocytes. It has been shown that their differentiation is strongly influenced by local environment and extracellular cues from such as Wnt produced by surrounding astrocytes. Microglia is a bone marrow-derived macrophage-like cell that plays an important role in brain immunity. We have previously found that microglia are localized adjacent to NSCs in the SGZ of the hippocampal dentate gyrus. This finding implies that, similarly to astrocytes, microglia can also affect the behavior of NSCs as a niche component. In epileptic conditions, activation of microglia occurs concomitantly with the increase in NSC proliferation, tempting us to hypothesize that NSC proliferation is increased by as yet unknown factors derived from microglia. To examine this hypothesis, we focus on Toll-like receptors (TLRs), since they have been known to activate microglia leading to the massive induction of gene expression of secreted factors. TLRs have been reported to be expressed in CNS cells, therefore we confirmed TLRs

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expression in the adult hippocampus by immunohistochemistry. We found that TLR9 is expressed in microglia, which reside in the SGZ. To reveal the role of TLR9 on NSC regulation in an epileptic condition, kainate was intraperitoneally administrated to wild type or *tlr9* knockout mice. We observed that the lack of TLR9 resulted in promotion of kainite-induced cell proliferation. As a next step, we sought to identify the proliferating cells in *tlr9* deficient mice following seizure induction with kainite by analyzing the phenotype of BrdU-labeled dividing cells. We found that the majority of these proliferating cells were neuroblasts, which are committed to differentiate into neurons. In addition, the number of newly generated mature neuron in the granular cell layer was significantly increased in *tlr9* knockout mice compared to that in wild type mice 4 weeks after kainite-induced seizure. This finding has prompted us to further examine whether behavior of NSCs are regulated by TLR9 expressed in microglia. *In vitro* studies indicated that microglia stimulated with TLR9 ligand released soluble factors that attenuated neuronal differentiation. Taken together, these data raise a possibility that TLR signaling in microglia regulates kainate-induced neurogenesis in the adult mouse hippocampus.

Poster Board Number: T-1055

SUPPRESSED CYTOKINE EXPRESSION IN NEONATAL RAT BRAINS IMMEDIATELY FOLLOWING TRAUMATIC BRAIN INJURY INDICATES A RAPID ENDOGENOUS ANTI-INFLAMMATORY RESPONSE

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The timing of therapeutic intervention in neurological disorders, especially neurotraumas such as traumatic brain injury (TBI) is critical (Glover et al., 2012 in press), with the acute phase already influenced by genetic and histologic perturbations including aberrant neurovascular inflammation and apoptosis (Borlongan and colleagues, 2009, 2010). Although immediate cell death cascades have become established in adult TBI, the pathophysiology underlying neonatal TBI is poorly understood. The objective of the present study was to determine the role of cytokine regulation following TBI in neonatal rats. Seven-day old Sprague-Dawley rats were subjected to TBI using the controlled cortical impact (CCI) injury model. Age-matched littermates that did not receive TBI served as the control for this study. Immediately following TBI (within 15 minutes), rats were euthanized and the brains were divided into the ipsilateral (left) and contralateral (right) hemispheres and flash frozen in liquid nitrogen. A BioRad 23-Plex panel was used to measure cyotkine levels. Surprisingly, the data revealed that 18 of the 23 cytokines analyzed were significantly downregulated in the hemisphere contralateral to the TBI impacted hemisphere. Three cytokines, namely, IL-5, IL-6 and MIP-3a were identified as significantly suppressed in both ipsilateral and contralateral hemispheres of neonatal TBI rats compared to the control rats. The remaining 5 cytokines did not signficantly differ between TBI and control rats, indicating an overwhelming downregulation of the entire panel of cytokines analyzed here. A parallel study processing the plasma of the same cohort of neonatal TBI and control rats revealed neither downregulation nor upregulation of the same cytokines analyzed in the brain tissue, suggesting a highly localized cytokine suppression in the brain during the early injury phase that was not reflected in the peripheral circulation. In stark contrast to the reported early pro-inflammatory response exhibited in adult TBI, the present neonatal TBI study demonstrated a reversed cytokine profile of downregulation. These results suggest a robust and immediate endogenous anti-inflammatory response is mounted by the contralateral hemisphere, a brain region remote from the site of injury. This study demonstrates that the highly plastic neonatal brain is equally capable of cytokine regulation following TBI, which may be a unique feature of the young brain's regenerative capacity.

Poster Board Number: T-1056

ADULT NEUROGENESIS AND NEURONAL REGENERATION IN THE ZEBRAFISH BRAIN

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The subventicular zone (SVZ) of the adult mammalian brain contains neural stem cells (NSCs) that generate neuronal progenitor cells (NPCs) to be supplied into the olfactory bulb (OB) in the physiological condition. However, brain insults such as ischemic stroke and traumatic brain injury stimulate NSCs in the SVZ to produce NPCs migrating to other brain regions for regeneration. The regenerative capability of such endogenous NSCs is insufficient to promote the functional recovery. To overcome this difficulty, we need to understand the mechanisms of adult neurogenesis and brain regeneration more precisely. The telencephalic ventricular zone (VZ) of adult zebrafish, in which powerful genetic approaches and *in vivo* imaging techniques are available, is an excellent comparative model to study the unsolved problems in neurobiology of the mammalian SVZ. We established a zebrafish model to study the cellular and molecular processes underlying adult neurogenesis and brain regeneration. Systematic immunohistochemical and ultrastructural studies of the adult zebrafish telencephalic VZ have revealed that its cytoarchitecture is similar to the VZ in the developing mammalian brain. Live imaging of fluorescently-labeled NPCs has shown that NPCs in the telencephalic VZ migrate along the blood vessels to the OB in adult zebrafish. Telencephalic injury induced coordinated cellular processes that underlie neuronal regeneration: the up-regulated proliferation of NPCs in the telencephalic VZ and the differentiated NPCs into mature neurons at the injury site. Eventually, the adult zebrafish brain appeared fully repaired within a month after the lesion. Moreover, we compared neurogenesis in the telencephalic VZ of young adult, mid-age, and elderly zebrafish. The number of NPCs migrating towards the OB was gradually decreased with age. The capacity for neuronal regeneration after telencephalon injury was also lower in aged fish. Clarifying the common and distinct mechanisms of adult neurogenesis, identified by the comparative studies of zebrafish and mammalian brains, may be useful to develop regenerative therapies for neurological diseases.

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GENE THERAPY USING WNT3A CONTRIBUTES TO FUNCTIONAL RECOVERY AND ENHANCES NEUROGENESIS IN FOCAL ISCHEMIC INJURY IN MICE

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Background: Stroke potently stimulates cell proliferation in the subventricular zone (SVZ) of the lateral ventricles. SVZ neuroblasts than migrate to the injured striatum and cortex; however, most of the cells do not survive and mature. Recent studies have demonstrated that Wnt signaling promote adult neurogenesis in the subgranular zone (SGZ) of the dentate gyrus in the hippocampus and the SVZ. However, it remains unknown whether overexpressing Wnt could promote neural regeneration in the striatum and functional recovery after ischemic injury. Here, we examined the efficacy of injection into the striatum or the SVZ of a lentivirus-expressing Wnt3a on neurogenesis and functional outcome following focal striatal ischemic injury induced by local administration of the vasoconstrictor endothelin-1. Results: Treatment with Wnt3a in the striatum significantly enhanced functional recovery after ischemic injury on the 28th day and increased the number of BrdU-positive cells differentiated into mature neurons in the ischemic striatum. Treatment with Wnt3a in the SVZ significantly enhanced the functional recovery from the second day after injury and increased the number of immature neurons in the striatum and the SVZ on the second day after injury. In addition, we found reduced disseminated neuronal injury in the group treated with Wnt3a in the SVZ. Conclusions: Our data suggests that gene therapy using Wnt3a contributes to functional recovery after ischemic injury, through increasing neurogenesis or neuronal survival in the ischemic striatum.

Poster Board Number: T-1058

CHARACTERIZATION OF EMBRYONIC STEM CELL-DERIVED NEURONS ON A HIGH-THROUGHPUT AUTOMATED PATCH CLAMP SYSTEM

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The advent of automated patch clamp systems has enabled the large scale screening of compounds for effects on ion channel activity through direct electrophysiological measurements. This type of platform technology can accelerate the drug discovery and development process for ion channel targets. However, the application of automated systems is currently limited to recording from cell lines that overexpress the ion channel of interest in a non-native context. In contrast, human stem cell-derived neurons (hSCNs) have shown promise to be a more physiologically relevant tool for drug discovery. Similar to primary neurons, hSCNs endogenously express ion channels in a more native-like context, enabling better pharmacological characterization of candidate compounds during drug discovery. To evaluate the suitability of hSCNs for automated patch-clamp electrophysiology studies, here we characterize the electrophysiological and pharmacological properties of endogenous ion channels in human embryonic stem cell (hESC)-derived neurons using an automated patch clamp system that measures both ligand and voltage-gated ion channels in 384 parallel recording sites. The hSCNs used for this study were derived from the WA09 (H9) hESC line and express a broad array of ion channels at the mRNA level but have not been characterized at a functional level. To date, we have demonstrated the functional expression of multiple voltage-gated ion channels in these hSCNs; a full biophysical and pharmacological characterization of these and other ion channel types expressed at the transcript level is underway. This study demonstrates that the combination of human stem cell-derived neurons with a high-throughput automated patch clamp system potentially offers a powerful assay for ion channel drug discovery in a more biologically relevant system.

Poster Board Number: T-1059

VOLUNTARY EXERCISE RESTORES THE SOCIAL RECOGNITION IMPAIRMENT IN DOMINANT NEGATIVE DISC1 MICE: IMPLICATION FOR SCHIZOPHRENIA

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Background: Schizophrenia (SCZ) is a severe neuropsychiatric disorder affecting about one percent of the population. Current pharmacological treatment options have insufficient effect on negative symptoms, such as social withdrawal, and cognitive decline. Clinical studies indicate that physical exercise can attenuate negative symptoms and improve cognitive deficits, possibly by enhancing adult neurogenesis. The aim of the present study was to determine whether voluntary wheel running can improve the SCZ-associated phenotypes of dominant-negative DISC1 transgenic mice (DN-DISC1). Results: DN-DISC1 and wild-type mice were placed for 14 days in an active cage with free access to a running wheel . Thereafter, mice underwent behavioral tests and brains were processed for tissue analysis. We could not observe differences in social interaction between the wild type and DN-DISC1 transgenic mice. Nonetheless, a significant deficit in social recognition was detected in the DN-DISC1 mutant mice which was markedly reversed by voluntary exercise in both females and males. We also found that female mice stayed in the active cage, restored the impairment observed in Y maze performance which is attributed to hippocampal dysfunction. ELISA analysis showed two fold increase in BDNF levels in frontal cortex of WT mice after physical exercise. In contrast, in the DN-DISC1 mice, physical exercise didn't induce the BDNF levels. Discussion: Our preliminary results suggest that physical exercise can attenuate the impaired social and cognitive phenotypes exhibited by the DN-DISC1 mice. In parallel, we observed no alteration in BDNF levels in the DN-DISC1 mice after physical activity which may indicate for their dysfunction in neurogenesis processes. Further tissue analysis of the brains will be conducted in order to reveal a possible mechanism underlying this behavioral data.

Poster Board Number: T-1060

LMX1A DEFINES MIDBRAIN DOPAMINERGIC NEURONS: FACS AND FICTION

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The derivation of specific neuronal subtypes from human pluripotent stem cells (hPSCs) at high purity is currently hampered by the inherent heterogeneity of existing differentiation protocols. Thus cell grafts may be contaminated with unwanted cell types, resulting in tumor formation and/or lack of functional integration. Isolating committed progenitors for use in both in vitro and *in vivo* applications is highly attractive since their reduced structural fragility when compared to post mitotic neurons, and proliferative potential allows for expansion prior to use. Previous work in this laboratory

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has demonstrated the isolation of committed mouse dopaminergic (DA) progenitors using a LIM homeobox transcription factor 1 alpha (Lmx1a) homologous recombinant reporter cell line. Depending on the culture method, the isolated Lmx1a+ progenitors give rise to enriched DA neurons in terminal cultures. In this study we investigate the potential of Lmx1a as a marker for the enrichment of DA neurons from hPSCs. We report that correct regionalisation of differentiating hPSCs during neural induction is required for the FACS isolation of committed floorplate (midbrain) DA progenitors using a human Lmx1a reporter cell line. These progenitors, when expanded as neurospheres and replated, give rise to enriched populations of functional bonafide midbrain DA neurons. Our findings show that it is possible to define and isolate cells suitable for either cell replacement therapies or the development of in vitro models of neurotoxicity.

Poster Board Number: T-1061

CULTURE, CHARACTERIZATON AND DIFFERENTIATION OF NEURAL STEM CELLS FROM GUINEA PIGS (CAVIA PORCELLUS)

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Neurogenesis in adulthood of most mammals persists in two main brain areas: the hippocampus dentate gyrus (DG) and the subventricular zone of the lateral ventriculum (SVZ). This study aimed to cultivate, characterize and differentiate neural precursors of fetal guinea pigs (*Cavia porcellus*). Culture of cell suspensions obtained from fetal SVZ were plated on *Poly-Hema* (Poly [2-hydroxyethyl methacrylate]) treated flasks and maintained in EGF and FGF-2-supplemented high glucose medium. After 7 to 10 days of culture, rounded, solid cellular aggregates with irregular surface and variable size were observed in the supernatant. These neurospheres (NSFs) were submitted to passages with or without enzymatic dissociation. Non-dissociated NSFs increased in size and number after passage whereas dissociated cells retained their proliferative capacity and eventually generated new NSFs. Dissociated and non-dissociated NSFs were frozen and thawed and the viability pre- and post-freezing, and after 1 to 15 days were maintained. The colorimetric method MTT revealed no differences in viability for thawed cells, examined soon after thawing or after 1 week or 1 month of thawing. In NSF cultures submitted to differentiation in EGF/FGF-2 deprived medium, a significant number of morphologically different, adherent cells were observed after 7 days. These cells were positive by immunostaining for markers of neural precursors (Nestin), neurons (β-III-tubulina), oligodendrocytes (mGalC) and astrocytes (GFAP). Thus, these results demonstrated the obtainance of NSFs with progenitor potential from SVZ of guinea pig fetuses.

Poster Board Number: T-1062

ESTABLISHMENT OF INDUSED PLURIPOTENT STEM CELL LINES FROM PARKINSON DISEASED PATIENTS FOR MOLECULAR MECHANISMS STUDY

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Parkinson's disease (PD) is the second most common neurodegenerative disorder (after Alzheimer's disease). Parkinson's disease is a gradually progressive, degenerative neurologic disorder. Although typically a sporadic disease, mutations in some genes have been identified as a cause of late-onset, autosomal dominant familial PD that is clinically and neurochemically indistinguishable from sporadic PD. Mutations in the leucine-rich repeat kinase 2 (LRRK2) are the most common cause of familial and sporadic PD identified to date. Mutation G2019S causes from 3% (Asia) to 41% (Northern Africa) of familial PD cases. Although some progress on LRRK2 involvement in the disease progression has emerged during last years absence of adequate model complicates investigation of human diseases and development of novel therapies. Recent advances in cell reprogramming technologies facilitate the development of human cell models that allows precise mechanisms disease investigation. Using skin biopsies from PD patients with LRRK2 gene mutation (G2019S) we reprogrammed dermal fibroblasts using lentiviral constructs carrying Yamanaka's factors (Oct4, Sox2, Klf4, c-Myc). Obtained iPSC clones were morphologically indistinguishable from human embryonic stem cells colonies. We analyzed PD patient-specific iPSC lines for the expression of main markers of pluripotency i.e. Oct4, Sox2, Hesx1, Sall1, TRA-1-60, TRA-1-81, SSEA4, etc. Established cell lines were also analyzed for the ability to differentiate into the cells belonging to all three germ layers. We developed an effective protocol of iPSCs differentiation into tyrosine hydroxylase - positive dopaminergic neurons. Generation of "diseased" iPSC lines from different patients carrying common genetic alterations allows use of phosphoproteomic approaches to identify possible molecular pathways of disease development and to find electrophysiological differences in neuronal network formation.

Poster Board Number: T-1063

NEURAL STEM CELLS DIRECTLY DIFFERENTIATED FROM PARTIALLY REPROGRAMMED FIBROBLASTS RAPIDLY ACQUIRE GLIOGENIC COMPETENCY

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Neural stem cells (NSCs) were directly induced from mouse fibroblasts using four reprogramming factors (Oct4, Sox2, Klf4, and cMyc) without the clonal isolation of iPSCs. These NSCs gave rise to both neurons and glial cells even at early passages, while early NSCs derived from clonal ESCs/iPSCs differentiated mainly into neurons. EGF-dependent neurosphere cultivation efficiently propagated these gliogenic NSCs and eliminated residual pluripotent cells that could form teratomas *in vivo*. We concluded that these directly induced NSCs were derived from partially reprogrammed

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cells, because dissociated ESCs/iPSCs did not form neurospheres in this culture condition. These NSCs differentiated into both neurons and glial cells *in vivo* after being transplanted intracranially into mouse striatum. NSCs could also be directly induced from adult human fibroblasts. The direct differentiation of partially reprogrammed cells may be useful for rapidly preparing NSCs with gliogenic competency.

Poster Board Number: T-1064

PHENOTYPIC SCREENING FOR NEUROGENESIS: FROM IN VITRO TO *IN VIVO*

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One of the most remarkable forms of structural plasticity in the adult brain is the generation of new functional neurons from adult neural precursors cells (NPCs). There is accumulating evidence that neurogenesis in the adult hippocampus contributes to brain physiology and disease, but its precise physiological role remains elusive. Conceptually, this process can be divided into four steps: (i) proliferation; (ii) neuronal fate determination; (iii) survival and neuronal/synaptic maturation of new neurons; and (iv) functional integration of new neurons into the pre-existing neuronal network. Here we describe the development of phenotypic *in vitro* screening assays using human embryonic stem cell derived NPCs as a cellular model to investigate neurogenesis. Using expression profiling of differentiating cells and exposure of NPCs to bioactive small molecules, we can demonstrate that neurogenesis relevant signaling pathways are active in this *in vitro* cell model. These experiments demonstrate that this human NPC model represents a neuronal relevant transcriptional and biological state, which can unleash the identification of a plethora of targets spanning the druggable genome. We identify through a screening cascade of High-Throughput Screening, image based High Content Analysis (neurite outgrowth, synaptic maturation) in human NPCs and subsequently in mouse model of adult hippocampal neurogenesis, new active CNS compounds/targets. These compounds may help in identifying novel regenerative medicines, and may ultimately elucidate novel mechanisms modulating adult neurogenesis.

Poster Board Number: T-1065

IN VIVO FATE AND FUNCTION OF NEURAL STEM CELL GRAFTS AND OF THE SUBSEQUENT ENDOGENOUS BRAIN IMMUNE RESPONSES

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In order to understand the physiological mechanisms needed for transplanted neural stem cells (NSC) to regenerate the injured central nervous system (CNS), we have aimed over the past years to determine NSC fate following in vivo grafting. For this, we established eGFP/Luciferase-expressing adherently growing cultures of murine embryonic (E14) brain-derived NSC, which were characterized by the expression of specific marker proteins and by their in vitro tri-lineage differentiation potential. Next, the therapeutic potential for intravenous administration of NSC was investigated in the experimental autoimmune encephalomyelitis mouse model for multiple sclerosis. The absence of therapeutic benefit was due to cell retention in lung capillaries, as demonstrated by real time *in vivo* bioluminescence imaging (BLI). Finally, we also investigated the *in vivo* remyelination capacity of NSC upon direct grafting into the demyelinated CNS of cuprizone-treated mice. At two weeks post-grafting, proper differentiation and integration of grafted NSC was hindered by strong microglial and astrocyte responses in both healthy and demyelinated CNS tissue, as demonstrated by quantitative *in vivo* BLI and histological analysis. Therefore, we hypothesize that the therapeutic potential of NSC grafting, as observed by other studies, cannot be accounted for by adequate cell graft migration, survival and/or integration, but may potentially be explained by induced inflammatory responses following cell grafting.

Poster Board Number: T-1066

A POTENTIAL APPLICATION OF OLFACTORY BULB-DERIVED ADULT NEURAL STEM CELLS

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Neural stem cells (NSCs), which are responsible for continuous neurogenesis during the adult stage, are present in human adults. Typical neurogenic regions are the hippocampus and the subventricular zone; recent studies have revealed that NSCs also exist in the olfactory bulb. Olfactory bulb-derived neural stem cells (OB NSCs) have the potential to be used in therapeutic applications and can be easily harvested without harm to the patient. Through the combined influence of extrinsic cues and innate programming, adult neurogenesis is a finely regulated process occurring in a specialized cellular environment, a niche. Understanding the regulatory mechanisms of adult NSCs and their cellular niche is not only important to understand the physiological roles of neurogenesis in adulthood, but also to provide the knowledge necessary for developing new therapeutic applications using adult NSCs in other organs with similar regulatory environments. We will present our recent progress of the study of adult NSCs regarding the potential of regenerative medicine, primarily in the area of diabetes therapy. The utility of NSCs derived from the olfactory bulb is presented along with the rationale for why this might be an acceptable treatment modality. The potential use of stem cells for the testing and recommendation of specific therapeutics for mental disorders is also presented.

Poster Board Number: T-1067

NESTIN-POSITIVE STEM/PROGENITOR CELLS WITH NEURAL DIFFERENTIATION POTENTIAL ARE PRESENT IN THE MENINGEAL NICHE DURING DEVELOPMENT UP TO ADULTHOOD.

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Meninges, which include arachnoid and pia mater, cover the entire CNS and are filled with cerebrospinal fluid produced by choroid plexi. Recently, we found that meninges host a population of immature precursors with neural differentiation potential that can be activated by injury. In this work, we asked whether these precursors in meninges were developmentally conserved from embryo to adult. Therefore, in samples from embryo (E14, E20), perinatal (P0, P15) and adult rats, we: i) quantify the distribution, the number and

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the proliferation rate of nestin-positive cells in meninges; ii) characterize the overall meningeal cell composition and its changes during the developmental stages; iii) assessed the stem/progenitor cells potential *in vitro*. The cellular and extracellular composition of meninges across development were analysed and the expression of markers of undifferentiated cell (Nestin and Vimentin), neural crest (p75), neural progenitors (Sox2, DCX, Tuj1), astorcytes (GFAP), pericytes (NG2), endothelial cells (CD31) and ECM components such as fibronectin, heparan sulphate and laminin, assessed by confocal microscopy. Cells expressing the stem cell marker nestin were found in meninges as early as E14. The number and density of these cells decreased with the animal age and represent the 13.3±4.4% of the adult rat meninges cells. Proliferating Ki67-nestin positive cells were found in all developmental stages even that their number significantly decreases (E20 16.5%, P0 10.9%, P15 8.7%6 and in adult 4.8%; $p<0.0001$). Moreover, Oct4- and DCX- positive cells were also found at all ages. Meninges nestin-positive cells were distinct from NG2-, GFAP- and CD31-positive cells. To further characterize meninges as stem cell niche, we analysed the gene expression of stem cell-related markers including nestin, pouf5, vimentin, cspg, dcx, pax6 and sox2 by laser capture microscopy. Quantification of the developmental changes of microdissected meningeal tissue confirm the persistence of stem cells-related gene during all the stages. Finally, we assessed the *in vitro* stem cell properties of meninges nestin-positive cells during development up to adulthood. As expected from the ex-vivo observation, the number of colony forming unit (CFU) and cell growth rate, decreased with rat age. Under differentiating conditions, nestine-positive cells expanded from all rat ages underwent neural differentiation. These data suggest that the meninges are a putative new stem cell niche capable of housing and maintaining up to adulthood a population of stem/progenitor cells with neural differentiation potential. Further investigation will elucidate any functional role of the meningeal stem cell niche and of its nestin-positive stem/precursor cells in brain development and in adulthood.

Poster Board Number: T-1068

EVIDENCE FOR NEUROGENESIS BY LATENT PARENCHYMAL PROGENITORS AFTER STROKE

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In the adult mouse brain, neurogenesis is limited to two small regions, one of which is the subventricular zone (SVZ) of the lateral ventricles. In response to stroke, neural stem cells in the SVZ increase their proliferation, and neuroblasts ectopically migrate toward the lesion site. Despite this well-described response, it is not known whether all stroke-induced ectopic neuroblasts are derived from the SVZ. It has been shown that following a cortical stab injury, parenchymal astrocytes, which are normally non-neurogenic, can be isolated and propagated as multipotent neurospheres in vitro. It has remained unknown whether parenchymal astrocytes can also display such neurogenic potential *in vivo*. To test this, we used Connexin-30-CreER transgenic mice or a Cre-expressing adenovirus to inheritably label parenchymal astrocytes, and subsequently subjected animals to experimental ischemic stroke through transient occlusion of the middle cerebral artery. We will present data providing evidence for stroke-induced local neurogenesis from parenchymal astrocytes *in vivo*. Our findings highlight a previously unknown level of plasticity in the adult brain and suggest a possible new target for therapeutic interventions that aim to repair the brain after ischemic injury.

Poster Board Number: T-1069

UNIQUE GENE EXPRESSION PROFILES OF NEURAL STEM CELLS AND THEIR PROGENY IN THE ADULT BRAIN

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Neural stem cells (NSCs) generate new neurons throughout life in two regions of the mammalian brain: the subventricular zone (SVZ) lining the lateral ventricles, and the hippocampal dentate gyrus (DG). Adult neurogenesis has been implicated in tissue homeostasis, physiologic brain function, and has been also associated with a number of neuro-psychiatric diseases. To characterize the molecular signature of NSCs and immature neurons we isolated NSCs and their progeny from the adult DG and SVZ using transgenic reporter mice expressing either a GFP reporter under the control of the Sox2 promoter (labeling NSCs), or a DsRed reporter under the control of the doublecortin (Dcx) promoter (labeling immature neurons). Transcriptome analyses revealed distinct gene expression profiles between NSCs and immature neurons. One example is the growth factor insulin-like growth factor 2 (IGF2), which was highly expressed in DG NSCs in contrast to SVZ NSCs and immature neurons. We show that IGF2 controls proliferation of DG NSCs in vitro and *in vivo* via the AKT pathway. Gene expression profiling of NSCs and newborn neurons may reveal novel genes/pathways that control distinct steps in the course of adult neurogenesis.

Poster Board Number: T-1070

NEURAL STEM/PROGENITOR CELLS DAMAGED BY REACTIVE OXYGEN SPECIES EVOLVED BY PHOTOSENSITIZING REACTION

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We want to know how the growth of neural stem/progenitor cells and their differentiation are affected by reactive oxygen species evolved in photosensitizing reaction, because of the similarity between the stem cells and the tumor cells in central nervous system. We investigated the effects of two photosensitizers (rhodamine 123 and hematophorphyrin) on the mouse neural stem/progenitor cells cultured *in vitro* under the illumination. ABC transporters were expressed in the cells, and could pump rhodamine 123 and hematophorphyrin out of the cells. Under the illumination of strong actinic light with those photosensitizers, reactive oxygen species was evolved to injure the cells. Number of viable cells decreased under illumination through apoptosis and necrosis. Those cell-killing activities were not clearly dependent on the presence of inhibitors for ABC transporters. Immunocytochemical staining with showed that immature cells with markers of neural stem/progenitor cells (Sox 2, CD133, nestin were more sensitive to the reactive oxygen species than the differentiated cells.

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Poster Board Number: T-1071

METABOLIC CONTROL OF ADULT NEURAL STEM CELL ACTIVITY

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Neurogenesis relies on an intricate balance between quiescence and proliferation of neural stem cells (NSCs) to ensure the lifelong generation of newborn neurons in the adult brain. The proliferative activity of NSCs is governed by highly regulated molecular mechanisms, many of which are yet to be identified. Using metabolic profiling of adult NSCs we observed that these cells are in a distinct metabolic state when compared to their differentiated progeny or other dividing neural cells. In particular, NSCs display high levels of *de novo* lipogenesis, a metabolic pathway upregulated in many cancer cells. Here we show the role key lipogenic enzymes play in maintaining NSC proliferation using pharmacological inhibition and conditional genetic deletion of these genes. Furthermore, we found that this pathway is tightly regulated in the hippocampal neurogenic niche through the specific expression of proteins which can inhibit the activity of these lipogenic enzymes within the quiescent pool of NSCs. Our findings underline the essential role of lipid metabolism in regulating the proliferative activity of adult NSCs.

Poster Board Number: T-1072

NEURONAL INDUCTION IN GRAFTS OF NEURONAL PROGENITORS TO SITES OF SPINAL CORD INJURY

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Keywords: spinal cord injury, neural stem cells pinal cord injury

(SCI) causes loss of neurons and axons at the lesion site, frequently resulting in permanent loss of function. One repair strategy is to use cell transplantation as a means of replacing injured neurons, providing a potential mechanism for forming poly-synaptic relays across sites of injury. The aim of the present study is to compare the fate of freshly isolated embryonic neural cells versus cultured, pre-dfferentiated neural cells that are transplanted into the injured adult spinal cord. Neural stem cells that express green fluorescent protein (GFP) were harvested from embryonic day 14 (E14) rat spinal cords and grafted into a C3 dorsal column wire knife lesion site in wild-type adult rats, 2 weeks after SCI. The grafts filled the injury site and matured into both neurons and glia; neurons extended axonal processes over long distances into the host spinal cord both caudally and rostrally. Fetal NPCs were generated from neurospheres derived from E14 spinal cord. When grafted into the adult injured spinal cord, these cells differentiated into glia. To increase neuronal differentiation/maturation from neurospheres, the cells were pre-differentiated into neuronal progenitors as monolayer cultures for 3, 5, 7 days using retinoic acid and sonic hedgehog. The cells expressed the early neuronal progenitor markers, III tubulin and Nestin. When grafting these pre-differentiated neuronal progenitors/early neurons into adult SCI lesion sites, almost no mature spinal cord neurons were detected in the cell graft 4 weeks after transplantation. Instead the graft consisted mainly of glial cells. Overall graft survival was good. These findings confirm the pro-glial environment of the adult spinal cord, indicating that even pre-differentiation into neuronal phenotypes are insufficient to lead to a neuronal fate *in vivo*. Further differentiation steps are required to promote retention of the neuronal phenotype in grafted neural progenitors. Such work is ongoing. ACKNOWLEDGMENTS: We thank the Rat Resource and Research Center, University of Missouri, Columbia, Missouri, for providing GFP rats. This work was supported by the Veterans Administration, NIH (NS09881), CIRM, Wings for Life, Canadian Spinal Research Organization, the Swiss Institute for Research into Paraplegia, and the Dr. Miriam and Sheldon G. Adelson Medical Research Foundation.

Poster Board Number: T-1073

MODELING PARKINSON'S DISEASE IN A PETRI DISH: EFFECT OF A- SYNUCLEIN ON DOPAMINERGIC NEURONS DERIVED FROM MOUSE INDUCED PLURIPOTENT STEM CELLS

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Parkinson's disease (PD) affects 1% of the population over the age of 60 and by the year 2030 the number of cases is estimated to double. PD is the second most prevalent neurodegenerative disorder. Lewy bodies are the hallmarks of PD pathology and accumulate the protein α-synuclein. α-synuclein overexpression is a causative factor for neurodegeneration as shown in Mendelian forms of PD. α-synuclein upregulation has also been consistently reported in toxicological animal models of PD. Several recent publications propose that α-synuclein can have a prion-like effect, where the disease state protein conformation can be transmitted between neurons. The disease relatively specifically targets A9 dopaminergic (DA) neurons in the midbrain and spares adjacent A10 DA neurons. In comparing these two populations in vitro we will explore the differences in susceptibility for PD. We hypothesize that A9 DA derived neurons will be more vulnerable to extracellular α-synuclein compared to A10 DA neurons in a new induced pluripotent stem cell (iPSC) model. We have generated iPSC lines by taking mouse tail fibroblasts and transfecting them with the four factors; specifically, Oct4, Sox2, Klf4 and l-Myc in a retroviral vector. The mouse tail fibroblasts are reprogrammed and iPSC colonies are picked after 21-30 days. Following characterization for pluripotency, the iPSCs are guided to a neuronal fate by using a novel four-stage protocol employing a feeder-free culturing method. Embryoid body formation is carried out in mESC media with the SMAD inhibitors Dorsomorphin (1 µM), SB431542 (2 µM) and the MEK inhibitor PD0325901 (1µM). After plating, the cells are cultured in N2/B27 media plus growth factors for 16 days and yield a significant percentage of beta III tubulin positive cells and tyrosine hydroxylase (TH) positive cells. Within the TH-positive population, A9 and A10 neurons can be distinguished using FoxA2 and calbindin markers, respectively. Challenging our iPSC-derived neurons with α-synuclein protein at different concentrations for 24 hours and subsequent flow cytometry and immunocytochemistry will illuminate the viability of A9 dopaminergic neurons versus adjacent A10 neurons. Comparing relative survival percentages of DA neurons can shed light on the inherent differences between A9 and A10 neurons. These differences are important when trying to understand why A9 neurons are more susceptible in PD. In addition, we believe that mouse dopaminergic neurons derived from induced pluripotent stem cells can be a valuable model of PD for 1) examining the characteristics of DA neuronal subtypes, 2) using these neurons for neurotoxicity

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testing, e.g. challenge with MPP+ and paraquat, and 3) establishing drug screening approaches. If deemed successful, our iPSC-derived neuronal model will be a valuable tool to study Parkinson's disease in a petri-dish.

Poster Board Number: T-1074

RFX TRANSCRIPTION FACTORS AND FGF1 SUSTAIN NEURAL STEM AND PROGENITOR CELLS THROUGH AURORA-A KINASE ACTIVATION

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Fibroblast growth factor 1 (FGF1) has been suggested as an important growth factor for many biological processes, such as neurogenesis. FGF1 and *FGF-1B* promoter-driven green fluorescent protein (F1BGFP) have been used to isolate neural stem/progenitor cells (NSPCs) with self-renewal and multipotency. However, the mechanisms that regulate the maintenance of F1BGFP(+) NSPCs are still unknown. In this study, we provide several lines of evidence to show the regulatory mechanisms for F1BGFP expression and the maintenance of F1BGFP(+) NSPCs: (i) RFX2 and RFX3 transcription factors could directly bind the 18-bp *cis*-element (-484 to -467) of F1B promoter, and are crucial for F1BGFP expression; (ii) F1BGFP(+) cells have significantly higher levels of AurA activation, neurosphere formation than GFP(-) cells. Protein kinase inhibitor, staurosporine, RFX2-siRNA and RFX3-siRNA could reduce *FGF-1B* promoter activity, AurA activation and neurosphere formation. These inhibitory effects could be rescued by FGF1 treatment. (iii) AurA kinase specific inhibitor, VX-680, could significantly reduce the AurA activation and neurosphere formation of F1BGFP(+) NSPCs. However, these reductions could not be rescued by additional FGF1 treatment. (iv) Autocrine/paracrine activation of AurA of F1BGFP(+) cells could be inhibited by FGF1-neutralizing antibody and FGFR (SU5402). Furthermore, AurA activation in F1BGFP(+) cells could also be inhibited by PI3K/AKT inhibitor, suggesting FGF receptors and AKT signaling pathways play important roles in the activation of AurA kinase and the maintenance of F1BGFP(+) NSPCs. (v) As a proof of principle, F1BGFP(+) cells are identified as a subset of B1 NSPCs with single primary cilium that will absorb through AurA activation in the lateral wall of the adult mouse brain. In conclusion, our results suggest that RFX2/3 transcription factors are crucial for F1BGFP expression levels. F1BGFP(+) cells have sustained AurA activation through FGF1-FGFR and PI3K/AKT pathways.

Poster Board Number: T-1075

P57 CONTROLS QUIESCENCE OF ADULT HIPPOCAMPAL NEURAL STEM CELLS

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Neural stem cells (NSCs) in the adult mammalian hippocampus constitute a relatively quiescent cell population, but the molecules responsible for their quiescence have remained unknown. In this study, we found that the cyclin-dependent kinase inhibitor Cdkn1c (p57) regulates the quiescence of NSCs in the adult mouse hippocampus. Selective deletion of the p57 gene in NSCs initially resulted in their recruitment into the cell cycle and increased neurogenesis in young adult mice and even in aged mice. In contrast, long-term deletion of the p57 gene through adulthood resulted in NSC exhaustion and a reduced level of neurogenesis in aged mice. Furthermore, our results support the notion that dynamic changes in p57 levels underlie the reversal of NSC quiescence in response to neurogenic stimuli such as epileptic seizures and running. The regulation of NSC quiescence by p57 might thus have important implications for lifelong neurogenesis and neural plasticity in response to external stimuli.

Poster Board Number: T-1076

MICRORNA-124 IS A NEURONAL FATE DETERMINANT IN THE SUBVENTRICULAR ZONE

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MicroRNAs are small, non-coding endogenous RNA that bind to and regulate mRNA targets, making them central players of gene regulation. miR-124 is one of the most abundant miRNA in the brain and several studies have shown that it plays a role in neural stem cell (NSC) regulation. To study the role of miR-124 in the postnatal subventricular zone (SVZ), we have generated a transgenic reporter mouse that allows visualisation of miR-124 activity in the brain *in vivo*. We found that miR-124 is not present in NSC, but its activity is initiated in transient amplifying progenitors and it remains expressed in differentiated neuronal progeny. When we stably suppressed miR-124 activity *in vivo* in the SVZ, adult neurogenesis was blocked and ectopic astrocytes, derived from the SVZ, were found in the olfactory bulb. On the contrary, when we over-expressed miR-124 in the SVZ we found that NSCs lost their stem cell characteristics resulting in a loss of continuous supply of new neurons to the olfactory bulb. In summary, our data provide novel insight into miR-124 function *in vivo* and positions miR-124 as a neuronal fate determinant in the postnatal SVZ.

Poster Board Number: T-1077

SYNTHETIC POLYMER-BASED NEURAL STEM CELL NICHE IDENTIFICATION

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FGF2 and Wnts are known to be secreted from microenvironments surrounding neural stem/progenitor cells (NSCs/NPCs) and act as mitogens and contribute to the maintenance of NSCs/NPCs. We have reported that these growth factors promote NSCs/NPCs proliferation via the common signaling pathway, ie, GSK3β inactivation, β-catenin accumulation, and cyclin D1 expression. We have also shown that this β-catenin accumulation results in potentiation of Notch signaling which leads to inhibition of neuronal differentiation (Shimizu et al., Mol. Cell. Biol., 2008). Furthermore, we recently found that cyclin D1, a downstream factor of FGF2 and Wnts, inhibited astrocyte differentiation through disturbing LIF/STAT3-mediated astrocyte-specific gene expression in a manner independent of cell cycle progression (Bizen et al., unpublished data.). Thus, the molecular mechanisms for NSC/NPC maintenance partly involve FGF2, Wnt, and Notch signaling pathways. NSCs are thought to require a specific niche for the maintenance of multipotency throughout a lifetime. However, despite the above mentioned findings, its molecular basis is poorly understood. It seems difficult to analyze the roles of the interaction between NSCs/NPCs and niche molecules, because of complexity of input stimuli from many niche components (such as ligands, cytokines, and ECM molecules) and NSC/NPC surface molecules. Although conventional approaches such as gene expression profiling and mass spectrom-

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etry analysis of NSCs/NPCs have been extensively done, the niche condition is still required to be studied. To solve this problem, we have established a polymer-based microarray system for screening NSC niche-mimicry. A total of 382 acrylate- and urethane-based polymers were printed on a standard 25×75 glass slide. NSCs/NPCs isolated from E14.5 mouse embryos were seeded on the polymer-printed slide. They attached strongly to 44 spot polymers. Among them, one acrylate-based polymer called "PA518" maintained a high level of nestin expression of NSCs/NPCs, in spite of the differentiation-inducing condition without FGF2. Furthermore, we found that the neurosphere-forming ability and multipotency of NSCs/NPCs plated on PA518-coated dishes was approximately 5-fold higher than those of NSCs/NPCs cultured on conventional poly-L-ornithine/fibronectin-coated dishes. Taken together, we here suggest that PA518 is a niche-mimicry molecule for NSC/NPC maintenance. Concurrently, we also screened glioma stem cell (GSC) niche mimicry and identified several hit polymers that supported the self-renewing proliferation of GSCs. These achievements may contribute not only to the understanding of NSC/GSC regulation but also to the development of novel therapeutic strategy for the regeneration medicine and cancer treatment in view of stem cells.

Poster Board Number: T-1078

EFFECT OF PLGA/SILK FIBROIN HYBRID FILM ON PROLIFERATION AND ATTACHMENT OF OLFACTORY ENSHEATHING CELLS

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Abstract: Poly (lactic-co-glycolic acid) (PLGA) has been widely applied to tissue engineering as a good biocompatible material and biodegradable synthetic polymer with acceptable mechanical strength. Silk fibroin contains powerful bioactive molecules and useful biomaterials in a range of applications. We fabricated silk /synthetic hybrid films using 0, 10, 20, 40 and 80 wt% of silk fibroin. Olfactory ensheathing cells (OECs) were seeded on PLGA/Silk fibroin hybrid film and confirmed the influence of adhesion and proliferation on OECs according to content of silk fibroin. Cell proliferation and viability were measured via MTT assay. In addition, morphology of cellular adhesion by scanning electron microscope (SEM). In this study, we confirmed PLGA/Silk fibroin hybrid film including 10% and 20% of silk fibroin interrupt adhesion and proliferation of OECs. In film containing 40% and 80% of silk provide suitable environment for growth and proliferation of OECs. This research was supported by WCU (R31-20029) and MBC (0405-B001-0204-0006). Keywords: Silk fibroin, Poly (lactic-co-glycolic acid) (PLGA), olfactory ensheathing cells WCU BIN Fusion Tech. Secretariat Dept. of BIN Fusion Tech., WCU, Eng. 8th Bldg. rm. No.408, Chonbuk National University, , 567 Baekje-daero, Jeonju 561-756 Korea Tel: +82 63 270 4434 begin_of_the_skype_highlighting +82 63 270 4434 end_of_the_skype_highlighting Fax: +82 63 270 4254 E-mail: bkwcu@jbnu.ac.kr

Poster Board Number: T-1079

IDENTIFICATION OF HDAC-MEDIATED MECHANISMS FOR THE TEMPORAL SWITCHING OF CORTICAL NEURON SPECIFICATION

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To date, the precise molecular mechanisms regulating the temporal switch in neural progenitor cells (NPCs) from producing deep- to superficial -layer neurons still remain unknown. On the other hand, increasing evidence suggests that epigenetic mechanisms play an integral part in NSCs fate specification. We have previously found that, histone deacetylases (HDACs) inhibition by valproic acid (VPA), a widely used anticonvulsant and mood stabilizing drug, drives mouse embryonic stem cells (mESCs)-derived NPCs to differentiate into Cux1-positive superficial-layer neurons at the expense of Ctip2-positive deep-layer neurons. Besides, *in vivo* HDAC inhibition *via* VPA treatment disrupted cortical laminarization with significantly increased and decreased thickness of Cux1-positive superficial- and Ctip2-positive deep-layers, respectively. Furthermore, our analyses on the effects of VPA on gene expression of cortical layer-specific transcription factors (TFs) demonstrated a down-regulation of *Fezf2*, a well-studied TF regulating deep-layer formation and up-regulation of *Cux1* and *Satb2*, TFs expressed by superficial-layer neurons. In this study, we further investigated the correlation between HDACs and NPCs fate specification during cortical neuronal differentiation, using mESCs and embryonic NPCs as models. The models are able to recapitulate *in vivo* cortical development while complementing and compensating for the limitations in respective model. Both cell types were treated with HDAC inhibitors and analyzed by immunocytochemistry, quantitative PCR and immunoprecipitation assay to elucidate the molecular mechanisms involved in cortical differentiation in response to HDAC inhibition. Our preliminary results suggest the important role of histone acetylation in cortical neuron specification especially in the specification of superficial-layer neurons during late corticogenesis.

Poster Board Number: T-1080

ASIALOERYTHROPOIETIN ENHANCES THE MATURATION OF OLIGODENDROCYTE PROGENITOR CELLS DERIVED FROM SUBVENTRICULAR ZONE AFTER NEONATAL WHITE MATTER INJURY.

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Perinatal hypoxia-ischemia (HI) frequently causes white-matter injury, leading to severe neurological deficits and mortality, and only limited therapeutic options exist. The white matter of animal models and human patients with HI-induced brain injury contains increased numbers of oligodendrocyte progenitor cells (OPCs). However, the origin and fates of these OPCs and their potential

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to repair injured white matter remain unclear. Here, using cell-type- and region-specific genetic labeling methods in a mouse HI model, we characterized the Olig2-expressing OPCs. We found that after HI, OPCs increased in the posterior part of the subventricular zone (pSVZ) and migrated into the injured white matter. However, their oligodendrocytic differentiation efficiency was severely compromisedcompared with the OPCs in normal tissue, indicating the need for an intervention to promote their differentiation. Erythropoietin (EPO) treatment is a promising candidate, but it has detrimental effects that preclude its clinical use for brain injury. We found that long-term post-injury treatment with a non-erythropoietic derivative of erythropoietin, asialo-erythropoietin (AEPO), promoted the maturation of pSVZ-derived OPCs and the recovery of neurological function, without affecting hematopoiesis. These results demonstrate the limitation and potential of endogenous OPCs in the pSVZ as a therapeutic target for treating neonatal white-matter injury.

Poster Board Number: T-1081

UNRAVELING THE FUNCTION OF A NOVEL SPLICE VARIANT OF NURR1 AND ITS IMPLICATIONS IN TAILORING MIDBRAIN DOPAMINERGIC NEURONS

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Recent work in the field of neuronal differentiation has given critical importance to generate midbrain dopaminergic (mDA) neurons from pluripotent stem cells and fibroblasts due to its physiological and clinical implications. Expression of the orphan nuclear receptor Nurr1 gene has been shown to be critical for the generation, survival and maintenance of mDA neurons and a key requirement for inducing mDA phenotype. Here, we report a splice variant of Nurr1 isolated from the mouse brain which lacks a part of its ligand binding domain (LBD), leading to in-frame deletion of 37 amino acids. Expression analysis of the splice variant named as Nurr1d, during embryonic (mouse) midbrain development revealed its differential temporal expression pattern. Interestingly, we found the mRNA expression of Nurr1d to be spatially overlapping with the expression of the *wildtype* Nurr1 only during the later differentiation stages of mDA neurons. Transient over expression studies on embryonic mouse ventral mesencephalon (VM) neural precursor's revealed that Nurr1 significantly enhances the number of tyrosine hydroxylase (Th) positive neurons compared to Nurr1d. Intriguingly, the Th positive neurons obtained from Nurr1d transfected cultures demonstrated a more matured neuronal phenotype with significant increase in neurite length and expression of late mdDA markers. To dissect possible distinct roles of Nurr1 and Nurr1d during mdDA development, we performed luciferase reporter assays using the Th and BDNF promoters on embryonic mouse VM primary cultures and secondary cell lines transfected with Nurr1 or Nurr1d. Here, we observed Nurr1 strongly potentiates the transcriptional activity of the Th promoter while in contrast Nurr1d significantly enhances the transactivation of the BDNF promoters. Furthermore, we investigated the neuroprotective ability of Nurr1 and Nurr1d transfected embryonic mouse VM cultures against the cytotoxic effects of 6-hydroxydopamine (6-OHDA). We found that the viability of mdDA neurons upon 6-OHDA challenge was significantly enhanced in cultures transfected with Nurr1d compared to Nurr1, demonstrating the robust neuroprotective potential of Nurr1d. In order to investigate the functional switch of the Nurr1d splicing variant on a structural level we are presently conducting differential nuclear magnetic resonance (NMR) analysis of Nurr1 and Nurr1d. Taken together our data indicate that Nurr1d attributing mdDA neurons with enhanced maturation and survival could be utilized in cellular differentiation paradigms aiming to obtain more mature and robust mdDA neuron phenotype.

Poster Board Number: T-1082

IDENTIFICATION OF A NEURAL INDUCTION DOMAIN IN BC-BOX PROTEINS AND NEURAL INDUCTION OF SOMATIC STEM CELLS BY TRANSFER OF ITS DOMAIN PEPTIDE

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Pluripotent somatic stem cells have potential to differentiate to neurons, and they are hopeful to be used as grafted donor cells for neuronal regenerative therapy. However, the grated cells little survive and differentiate to functional neurons in recipient neural tissue. To overcome the problem, neural induction using neurotrophic factors or gene transfer has been employed before grafting, but neurotrophic factors do not occur specific neural induction, while gene transfer has risk of vector. If a neural induction domain for somatic stem cells exists in proteins to induce neural differentiation, its identification can contribute to neuronal regenerative therapy through neural induction of somatic stem cells using the neural induction domain. We previously demonstrated that von Hippel-Lindau tumor suppressor (VHL) protein has a function of neural induction in neural stem cells (NSCs) without any neurotrophic factors. Then, we hypothesized that a neural induction domain potentially exists in the VHL protein. Here we identify a neural induction domain for somatic stem cells in the VHL protein, and show neural induction of the cells by transfer of the domain peptide linked to protein transduction domain (PTD). The neural induction domain in the VHL protein contains BC-box motif [(A,P,S,T) LXXX (A,C) XXX(A,I,L,V)] corresponding to binding site of elongin BC, which is evolutionally conserved from virus to mammalian. Therefore, we proposed that other BC-box proteins also contain the neural induction domain, and subsequently show to identify the neural induction domains at amino-acid sequences encoded by BC-box motif within BC-box proteins responsible for neural induction of somatic stem cells. In addition, we show that the domain has the same function for other somatic stem cells except for neural stem cells. Furthermore, when the domain peptide-transferred stem cells are grafted into recipient nervous tissue in neuronal disease models, the grafted cells differentiate to neurons and neuronal repair for neuronal disease models is achieved. Thus, a neural induction domain is identified at BC-box motif in BC-box proteins. The neural induction of somatic stem cells is caused by transfer of the neural induction domain peptide linked to PTD, and would contribute to neuronal regenerative therapy.

Poster Board Number: T-1083

HEAT-SHOCK PROTEIN 27 PROTEIN IS DOWNREGULATED IN THE PROCESS OF PLACENTA-DERIVED MULTIPOTENT CELLS (PDMCS) DIFFERENTIATED INTO NEURON

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Placenta-derived multipotent cells (PDMCs) isolated from the human placenta are a population of multipotent cells that are able to differentiate into multiple cell types. It is well known that1-methyl-3-isobutylxanthine (IBMX) induces the differentiation of PDMCs

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or mesenchymal stem cells into neural cells. Using proteomic approaches we found the small heat-shock protein 27 (HSP27) was downregulated at the transcriptional and translational levels during IBMX-induced neuron differentiation in PDMCs model. Overexpression of HSP27 in PDMCs led to the arrest of specific neural differentiation; On the other hand, knockdown of HSP27 using short-hairpin RNA showed significantly enhanced ability of PDMCs differentiated into neuron. Multiple neuronal markers were also stained to confirm the identity of differentiated neuron including MAP2, Tuj1, Tau, Neuron D and NFM. We conclude that downregulated HSP27 protein is a crucial factor in the differentiation of PDMCs into neurons. These findings provide new insights into the neuronal differentiation of PDMCs.

Poster Board Number: T-1084

THE MOOD STABILIZERS VALPROATE AND LITHIUM ACTIVATE HUMAN FGF1 GENE 1B PROMOTER THROUGH RFX TRANSCRIPTION FACTORS

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Valproic acid (VPA) and lithium are two primary mood-stabilizing drugs to have neuroprotective effects and be used to treat bipolar disorder in clinic. Fibroblast growth factor 1 (FGF1) has been shown to regulate cell proliferation, cell division and neurogenesis. Human *FGF1* gene 1B promoter (-540 to +31)-driven green fluorescence (F1BGFP) was shown to recapitulate endogenous *FGF1* gene expression. It can also be used to isolate neural stem/progenitor cells (NSPCs) from developing mouse brains and human glioblastoma tissue. Our previous study showed that transcription factors RFX2 and RFX3 could directly bind the 18-bp *cis*-element (-484 to -467), and contribute to the maintenance of F1BGFP(+) NSPCs. In this study, we showed that treatment of human glioblastoma cell lines with VPA and lithium significantly elevated the levels of FGF1B transcript and the percentage of F1BGFP(+) cells in both FGF1B(+) and (-) cell lines. Interestingly, co-treatment of lithium with VPA could synergistically activate F1BGFP expression. The increase of F1BGFP expression level by VPA treatment was associated with induction of neuronal differentiation. Upon VPA treatment, the F1BGFP(+) cells were differentiated more efficiently into MAP2- and tyrosine hydroxylase-positive neurons than F1BGFP(-) cells. Meanwhile, VPA treatment also sustained the expression levels of neuronal lineage specific transcription factors, NeuroD1, Myt1L, Brn2 and Ascl1 in F1BGFP(+) cells. Using electrophoretic mobility supershift assay and Western blot, we found that treatment of VPA promotes acetylation of RFX2/3 complex. In addition, VPA or lithium treatment also significantly increased mRNA levels of RFX2 or RFX3. Notably, knockdown of RFX2 could significantly attenuate VPA-enhanced GFP expression in F1BGFP(+) NSPCs cells. This study suggested, for the first time, that FGF1 is the target for VPA and lithium. Our results provide valuable implication for therapeutic application of these two mood stabilizers.

Poster Board Number: T-1085

MULTIVALENT EFFECTORS TO CONTROL STEM CELL DIFFERENTIATION

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Numerous cellular signaling systems that regulate stem cell self-renewal and differentiation involve the assembly of multivalent ligands - oligomeric entities that present multiple binding sites and thereby bind multiple receptors on a target cell or stem cell - such as morphogens that naturally oligomerize, extracellular matrix protein engagement of integrins, and juxtacrine signaling between membrane-associated ligands and receptors. The resulting multivalent binding can be more potent than corresponding monovalent interactions, potentially through initiating a process of cellular receptor clustering that may promote enhanced signal transduction. However, the ability to control and monitor these naturally occurring multivalent interactions within a living cell is currently limited, since it is difficult to modulate the valency of naturally-occurring ligands, especially membrane-associated proteins. We have developed a means to synthetically conjugate the binding domains of normally cell membrane-associated ligands to the soluble biopolymer hyaluronic acid (HA). Specifically, using EDC/NHS chemistry, we functionalized the high molecular weight HA backbone and attached recombinantly produced ephrin-B ligand ectodomains via an added terminal cysteine. To precisely estimate the valency and molecular weight of the bioconjugates, we characterized the molecules using size exclusion chromatography and multi-angle light scattering. To determine the effects of bioconjugate multivalency on the ability to cluster cell surface receptors, we incubated adult hippocampal neural progenitor cells (AHNPCs) with the multivalent molecule in vitro. Multivalent ephrin-B showed enhanced clustering of cognate Eph receptors as compared to antibody-clustered ligands. Next, to determine if increased receptor clustering could affect stem cell differentiation, cultures of AHNPCs were incubated solely with either antibody-clustered ligands or high conjugation ratio conjugates for six days. A maximum three-fold increase over the antibody-clustered ligand for the highest ratio conjugate was observed. To determine if the effect of multivalency in directing neuronal stem cell fate could be applied more generally to other cell types, cultures of human embryonic and induced pluripotent stem cells were incubated with the factors for 2-4 weeks. In both cultures a 2-fold increase in the fraction of neurons formed and a 4-fold increase in the fraction of dopaminergic neurons compared to antibody-clustered controls was observed. Cultures also showed increases in the midbrain-specific marker En1 and had significantly higher levels of dopamine. Finally, in an effort to validate the enhanced activity of this multivalent construct *in vivo*, we injected it directly into the brain of adult rats via intracranial stereotaxic injection. Brains injected with bioconjugates showed a three-fold increased ability to induce neuronal differentiation as compared to the antibody-clustered control, thus indicating that highly multivalent ligands potently enhance the fraction of new neurons formed compared to using ligands clustered by conventional means. These results have a variety of biomedical implications, in that they establish a general platform for creating highly bioactive, defined, and reproducible forms of protein-based ligands, which have the ability to more potently activate downstream effectors for use in basic research and therapeutic applications.

Poster Board Number: T-1086

EXPRESSION OF TYROSINE HYDROXYLASE IS EPIGENETICALLY REGULATED IN NEURAL STEM CELLS

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Tyrosine hydroxylase (TH) is the first and rate-limiting enzyme in biosynthesis of catecholamines. TH expression is regulated in a developmental stage and cell type-specific manner. We have previously reported that the elements responsible for cell type-specific

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expression of TH may be included within the repressor region between -2187bp and -1232 in TH promoter. To unravel the underlying molecular mechanisms for this specificity, DNA methylation patterns of CpG islands in the repressor region of TH promoter were examined in neural stem cells and DA neuron-like cells. Using a bisulfite sequencing method, we found that cytosine residues of CpG island within NRSE-R site was specifically methylated in human NSC, but not SH-SY5Y cells. A good correlation was observed between this CpG methylation and lower expression of TH gene, which was supported by the data that inhibiting DNA methylation with 5-azacytidine restored TH expression in neural stem cells. We further demonstrate that methylated CpG binding proteins (MBDs) actually binds to the highly methylated X-1 and X-2 regions of TH gene in neural stem cells. These results suggest that region-specific methylation and MBDs play an important role in the regulation of hTH gene in NSCs.

Poster Board Number: T-1087

INJURY-ACTIVATED NESTIN AND DCX POSITIVE CELLS IN MENINGES CONTRIBUTE TO GLIAL SCAR FORMATION FOLLOWING SPINAL CORD INJURY

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Adult spinal cord has little regenerative potential, thus limiting patient recovery following injury. We have previously described stem/progenitor cells with neural differentiation potential in postnatal rat brain located in the meninges (arachnoid and pia mater) covering the parietal cortex. Based on this finding we hypothesize that adult spinal cord meninges may be a niche for a neural stem cell-like population. In this study we describe a new population of cells resident in the adult rat spinal cord meninges that express the neural stem/precursor markers nestin and doublecortin. A stem/precursor cell population was extracted from dissociated meningeal tissue, cultured in vitro as neurospheres up to several month and subsequently differentiated into functional neurons and mature oligodendrocytes. We also provide evidences of participation of meninges-derived stem/precursors cells to the parenchymal reaction to injury. Indeed we observed that the proliferation rate and number of stem/precursor cells in meninges increased *in vivo* following spinal cord injury. By using a lentivirus-labeling approach, we followed the migration of the meninges-derived stem/precursor cells into the parenchyma in injured animals and we found that they contribute to the glial scar formation. Our data highlight the multiple roles of meninges in the reaction of the parenchyma to trauma and indicate for the first time that spinal cord meninges are potential niches hosting stem/precursor cells that can be activated by injury. Meninges may be considered as a new endogenous source of adult stem/precursor cells to be further tested for use in regenerative medicine applied to neurological disorders, including repair from spinal cord injury.

Poster Board Number: T-1088

NEUROSPHERE ATTACHMENT AND ASTROGENESIS ARE INDUCED BY BMP4 VIA PI3K MEDIATED UPREGULATION OF N CADHERIN

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Neurospheres are three-dimensional free-floating aggregates of neural stem cells (NSCs). These structures have been used as culture systems for the expansion and differentiation of NSCs without affecting self-renewal potential and multipotency. The formation of neurospheres is affected by various factors such as epidermal growth factor (EGF) or Fibroblast growth factors (FGF) - 2 which are maintain the self-renewal characteristics. However, the extrinsic signals that affect the formation or dissociation of neurospheres are poorly understood. In this study, we found that bone morphogenetic protein 4 (BMP4) induces astrocytic differentiation and migration of neurosphere NSCs through the attachment of neurospheres. These effects were accompanied by Akt activation and N-cadherin upregulation whish is the adhesion molecule. PI3K inhibitor blocked the attachment of neurosphere, astrocytic differentiation and N-cadherin upregulation of neurosphere NSCs. Especially, BMP4-induced neurosphere attachment, astrocytic differentiation, and migration of neurosphere NSCs were inhibited by neutralizing N-cadherin antibody. Together, these findings show that BMP4-induced attachment of neurospheres is related to the astrocytic differentiation of these cells and that these effects are attributable, at least in part, via PI3 kinase-Akt pathway-dependent induction of N-cadherin.

Poster Board Number: T-1089

THE EFFECT OF LEUKOENCEPHALOPATHY VANISHING WHITE MATTER MUTATIONS ON GLIAL DIFFERENTIATION

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Vanishing white matter disease (VWM) is an autosomal recessive leukoencephalopathy caused by mutations in genes encoding the eukaryotic translation initiation factor 2B (eIF2B). Children are mainly affected, who suffer from progressive neurological symptoms as ataxia and die at young age. Previous research has shown that astrocytes and oligodendrocytes in their brain white matter show a defect in maturation. Currently there is no treatment available. Our research is focused on 1) To test prospects for cell replacement therapy for VWM patients. Transplantation of stem cells in early stages of the disease may halt further progression of the disease and repair the existing damage. We have generated mouse models for VWM, which show a phenotype that is similar to that of the human patients. These mice offer us excellent models to test cell replacement therapies. 2) To investigate *in vitro* the capability of VWM stem cells to differentiate into fully mature astrocytes and oligodendrocytes. We use induced pluripotent stem cells (iPSCs) derived from VWM mice and human patients, and test the effects of a VWM microenvironment on the differentiation of oligodendrocytes. By combining *in vitro* and *in vivo* stem cell work, we hope to elucidate the VWM disease pathology further and develop new insights into therapeutic strategies for VWM and childhood white matter disorders in general.

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MOTOR NEURONS AND DOPAMINERGIC NEURONS: NOVEL STEM CELL-BASED SOLUTIONS ACCELERATING RESEARCH AND DRUG DISCOVERY

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Neurons isolated from human tissue have been widely used in basic research, drug discovery and for treatment of neurodegenerative disorders. However, the use of these cells is mired in ethical and technical issues mainly associated with procurement of human tissue and isolation of functional cells from the tissue. Alternatively, human pluripotent stem cells (hPSCs) including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) offer great promise to generate specialized cells from different lineages including human neurons. Lonza, Inc. in partnership with California Stem Cell, Inc. recently launched its first hESC-derived cell product - MotorPlate™, which are functional, high-purity human motor neurons (hMNs). MotorPlate™ hMNs are available in a ready-to-use format (96-well plates or 384-well plates) for use in high throughput applications. We further developed a method to cryopreserve hMNs and demonstrated comparable characteristics to non-cryopreserved neurons. The frozen vial format provides more flexibility to researchers in the field. In early 2012 Lonza, Inc. entered into a partnership with xCell Sciences, LLC with the intention to provide dopaminergic (DA) neurons derived from hESCs and hiPSCs to researchers in academia and industry. Following differentiation, the majorities of cells are Tyrosine Hydroxylase- (TH-) positive and co-express midbrain and A9 dopaminergic markers. Transplantation of these cells in 6-hydroxydopamine induced parkinsonian rats resulted in amelioration of behavioral deficits, demonstrating the functionality of these DA neurons. DA neurons derived from hESCs will be offered in a high throughput format (96-well and 384-well plates) as well as cryopreserved vials. In addition Lonza will offer generation of DA neurons from iPSCs on a fee-for-service basis upon request. In conclusion, Lonza, Inc. has established itself as a reliable source of functional and high purity Dopaminergic and Motor Neurons suitable for use in basic research, drug discovery and toxicity testing.

Poster Board Number: T-1091

RECEPTOR-SPECIFIC BLOCKING OF NOTCH SIGNALLING

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The Notch signalling pathway is instrumental for cell fate diversification at multiple stages in development and in cell proliferation in both normal and diseased states. The pathway has been studied extensively in mammalian neural stem cells both *in vivo* and in vitro; however there is still much uncertainty about the specific functions of individual Notch receptors and ligands. In mammals there are 5 ligands, which are capable to promiscuously activate the 4 different Notch receptors (Notch 1-4). Despite this promiscuity, individual family members have been associated with distinct roles in fate regulation and also to exhibit different expression pattern in various contexts. Thus the ability to specifically block or activate individual Notch receptors would provide greater understanding of the role of the individual receptors and would permit a greater degree of control in model systems, e.g. stem cells. From a scFv phage display antibody library we have selected and characterised antibodies specifically binding to Notch family members including receptors Notch 1-3 and the ligands Jagged and Delta. Further, we have identified blocking antibodies for Notch1 and Notch2. The potency of blocking antibodies has been determined in a co-culturing assay and antibodies targeting Notch 1 and 2 are silencing signalling completely. By qRT-PCR we have verified that downstream target genes of Notch are down regulated when blocking antibodies are added to neural stem cells in vitro. Functional antibodies provide a route to block or activate the individual Notch receptors. In contrast to other methods, this could be done reversible in genetically unmodified cells or animals and thus enable flexible regulation of signalling at multiple stages.

Poster Board Number: T-1092

GENERATION OF PATIENT-SPECIFIC INDUCED PLURIPOTENT STEM CELLS CARRYING MUTATION IN SOD1.

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Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disorder in which motor neuron (MN) loss in the spinal cord and motor cortex leads to progressive paralysis and death. Although most cases are sporadic, about 10% of cases are inherited. Super oxide dismutase 1 (SOD1) is the first identified gene which causes ALS. Accumulating evidence, explored by ALS mice model expressing mutant SOD1, provides several hypotheses. However, there is no effective cure so far. To establish drug screening platform, it is necessary to reveal cellular phenotypes in human MNs and other types of cells. Here, we describe the generation of induced pluripotent stem cells (iPSCs) from 6 familial ALS patients and the differentiation of disease-specific iPSCs into MNs to establish an ALS model *in vitro*. To generate patient-specific iPSCs, dermal fibroblasts were obtained by biopsy from patients carrying mutant SOD1. The fibroblasts were reprogrammed by retrovirus vectors or episomal vectors. These iPSC lines expressed ESC markers such as Nanog and SSEA-4, and had pluripotency to differentiate into cells of all three germ layers *in vitro* and *in vivo*. DNA sequencing revealed that mutated alleles of iPSCs are identical to the original specimen. All iPSCs preserve normal karyotype. Disease-specific iPSCs were differentiated into MNs expressing HB9 and ChAT. These results indicated that disease-specific iPSCs might provide an ALS model system *in vitro*.

Poster Board Number: T-1093

INHIBITION OF CRUCIAL TUMOR SUPPRESSOR PATHWAYS IN ADULT NEURAL STEM CELLS RESULTS IMBALANCED HOMEOSTASIS AND GLIOMAGENESIS

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Neural stem cells (NSCs) have been suggested as cellular origin of brain tumors. How the endogenous NSCs contribute to brain tumorigenesis instead of neurogenesis remains poorly understood. We have previously shown that the nuclear receptor Tlx is crucial for brain tumor initiation from adult NSCs. Here we show that the tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome 10) is inhibited by Tlx in the adult NSCs. Inactiva-

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tion of the PTEN gene specifically in type B cells of the adult mouse subventricular zone (SVZ) causes transient expansion, but surprisingly a long-term decrease of neural stem/progenitor cell proliferation. We provide evidence that an increase of cellular senescence contributes to the loss of PTEN-deficient neural stem/progenitor cells. p21 is up regulated in the PTEN mutant but down regulated in Tlx overexpressing NSCs. Genetic ablation of p53, which leads to a down regulation of p21, together with PTEN loss leads to a continuous increase in stem cell proliferation and brain tumor formation. In addition, we observed a critical cell fate switch of the mutant NSCs, which demonstrate the redirection of cell fate toward glial lineage is one important step for glioma development. This study unmasks a core pathway in regulation of the balance between adult NSC homeostasis and tumorigenesis.

Poster Board Number: T-1094

IN VIVO IMAGING OF ENGRAFTED NEURAL PRECURSOR CELLS: ITS APPLICATION IN EVALUATING THE OPTIMAL GRAFT CELL NUMBER FOR SPINAL CORD INJURY

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Neural precursor cells (NPCs)-based approaches have been acclaimed as potential treatments for many neurodegenerative disorders, including spinal cord injury (SCI). Indeed, there are a number of reports in which the transplantation of NPCs in animal models of SCI has been shown to improve the functional recovery, and some clinical trials using NPCs have also been performed or initiated. However, the transplantation protocols are completely different in several ongoing clinical trials for spinal cord injury (SCI) with cell therapy and, in particular, there is a considerable variation in the number of transplanted cells (ranging from 2e5 to 3.7e8 cells), which is exactly the same situation as in animal experiments (ranging from 7.5e4 to 12.5e6 cells). This chaotic situation hampers the establishment of an optimal treatment for SCI. Nevertheless, there has been no attempt to determine the optimal number of cells for transplantation because of the lack of suitable methodology. Conventional evaluation to quantify the number of engrafted cells mainly relies on histological examination, in which it is very difficult to distinguish living or dead cells and to count all cells. Therefore, we employed a bioluminescence imaging (BLI) system to address this question. NPCs were harvested from embryonic mouse striatum and labeled with luciferase and green fluorescent protein (GFP) reporter genes via lentiviral transduction, and GFP-positive cells were expanded with a neurosphere assay. To examine whether the number of transplanted cells affects cell survival and functional improvement, different numbers of lentivirally labeled NPCs (1×e5, 2.5×e5, 5×e5, or 1×e6 cells) were transplanted into the injured spinal cord immediately after contusion injury at the 10th thoracic level. After confirming that the number of transplanted NPCs correlated with bioluminescence intensity, we tracked the bioluminescence intensity and observed comparable rates of NPCs survival, irrespective of the number of transplanted NPCs. All NPCs-transplanted groups showed better locomotor function recovery than the medium-injected control group in two open-field motor scores (Basso, Beattie, and Bresnahan (BBB) score and Basso Mouse Scale (BMS)) and footprint analysis. However, functional recovery was not significantly different among the NPCs-transplanted groups. Correlational analysis revealed no relationship between the number of surviving NPCs and the subsequent functional recovery at any time point. Furthermore, we transplanted a broader range of NPCs (ranging from 2.5×e4 to 2.5×e6 cells). The transplantation of 2.5×e4 NPCs did not significantly affect functional improvement, but that of 2.5×e6 NPCs had a negative effect on functional improvement. These results indicate that optimizing the number of graft NPCs is crucial for the treatment for SCI.

Poster Board Number: T-1095

PROTOCOLS FOR MATURE MOTOR NEURON ISOLATION

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This study aims to present motor neuron (MN) isolation protocols that will allow a sufficient yield of cells for analysis by means of immunohistochemistry, in situ hybridization, quantitative polymerase chain reaction (qPCR), and microarray analysis. Recent advances in stem cell neurobiology have allowed the production of MNs from stem cells using appropriate signaling factors such as retinoic acid (RA) and sonic hedgehog (Shh). However, obtaining a uniform population of MNs in cell culture has been a challenge, as stem cells differentiate into other neural cell fates. Obtaining a homogenous MN population has also been a challenge *in vivo*, as a species ubiquitous protocol for isolating MNs from the spinal cord has yet to be developed. This study aims to describe how a uniform population of MNs can be isolated in vitro and *in vivo* using isopycnic density centrifugation and fluorescent activated cell sorting (FACS). Hb9::GFP transgenic mouse embryonic spinal cords were dissected from 14.5 day old embryos. Liberase dissociated spinal cords were subjected to FACS using the homeobox gene Hb9::GFP reporter and neurotrophin receptor (p75NTR) antibody as MN isolation markers. FACS assessment indicated an isolation of 31.2% Hb9::GFP and p75NTR double positive *in vivo*-derived spinal cord MNs. Liberase dissociation of day 8 Hb9::GFP mouse embryonic stem cell (mESC)-derived MNs were also subjected to FACS, showing an isolation of 61.3% Hb9::GFP and p75NTR double positive in vitro-derived MNs. qPCR analysis targeting MN specific markers such as choline acetyltransferase (ChAT) and LIM homeodomain protein islet-2 (Isl2) showed higher normalized ratios of ChAT and Isl2 expression in Hb9::GFP and p75NTR double positives. Papain dissociated e14 mouse spinal cords were subjected to a HistoDenz density gradient. Immunocytochemical cell counts of Hb9::GFP positive cells showed a 2.73% yield of MNs. From FACS analysis and HistoDenz density centrifugation, we can conclude that MNs were isolated, albeit at a moderate efficiency using FACS and low efficiency using density centrifugation. Isolation of homogenous MN populations in vitro and *in vivo* is essential; as a comparison of these two systems will provide information on how accurately stem cell-derived MNs resemble their *in vivo* complement. Analysis of isolated MNs via in situ hybridization and microarray will allow identification of new key regulators responsible for MN development; aside from already known common regulators. This feat will allow the development of a defined in vitro model for neurodegenerative diseases, as well as offer hope for future cell replacement therapy.

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SMALL MOLECULES THAT INDUCE NEUROGENESIS AND INHIBITS GLIOGENESIS IN NEURAL STEM CELLS

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Human neurodegenerative disorders are closely associated with a loss of neurons and glial cells in the central nervous system. Neural stem cells (NSCs) are potential and attractive sources for cell replacement therapy and regeneration in the central nervous system (CNS), and small molecules that can modulate NSCs differentiate into a certain cell types can be useful agent for the therapeutic application. In an effort to provide stem cell-based therapeutic approaches using NSCs, an *in vitro* screen was performed in search of chemicals inducing neurogenesis in NSCs. About 100 chemicals were screened through image based screening, and we found the chemical named KHN 01, that significantly induce neurogenesis and inhibited gliogenesis. KHN 01 and its derivatives, KHN 02 and 03, were tested to see if they could regulate NSC differentiation. Immunocytochemistry analysis showed that KHN 01, 02 and 03 induced neuronal marker βIII tubulin and inhibited production of glial cells such as astrocytes and oligodendrocytes, suggesting that KHNs selectively induce a neuronal differentiation in expense of gliogenesis. Immunocytochemistry analysis using antibodies against BrdU and Ki67 revealed that KHNs inhibited cell proliferation. The neurogenic effects of KHNs disappeared when epithermal growth factor was present. In addition, KHNs synergistically increase neurogenesis when treated with PD 98059, a selective MEK inhibitor. From these observations, KHNs reduced cell proliferation and induced neurogenesis along with the inhibition of gliogenesis in NSCs, and may be useful for modulating NSC fate.

Poster Board Number: T-1097

IDENTIFICATION OF MOLECULES IMPORTANT FOR THE DEVELOPMENT OF RESPIRATORY MOTOR CIRCUITS USING MICROARRAY ANALYSIS

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Respiratory failure is the most common cause of death in motor neuron (MN) degenerative diseases such as spinal muscular atrophy and amyotrophic lateral sclerosis and in upper cervical spinal cord injuries. The advent of methods to generate spinal MN from both embryonic and induced pluripotent stem cells has opened up new possibilities for studying the pathogenesis of MN disease *in vitro* and developing cellular therapies to replace damaged neurons and restore motor functions. However, most of the procedures currently used to generate MNs from stem cells produce only a limited subset of the MN classes found *in vivo* and do not efficiently generate respiratory MNs. To overcome this challenge, we have set out to define the molecular pathways that lead to MN formation and functional diversification. While much emphasis has been focused on understanding the development of limb-innervating lateral motor column (LMC) MNs, the factors important for hypaxial/respiratory motor column (HMC) specification remain poorly defined. We have previously demonstrated that the transcription factor *Foxp1* plays an essential role in LMC MN formation by suppressing the generation of HMC MNs. To identify novel determinants of HMC MN fate, we have carried out gene expression profiling experiments comparing control and *Foxp1* mutant MNs. This analysis has revealed a number of transcription factors, growth factors, and axon guidance molecules present in HMC MNs that may serve a critical function in respiratory MN development and circuit assembly. We will present our current research into the molecular and functional analyses of the genes unveiled in this study.

Poster Board Number: T-1098

ABERRANT FOCAL ANGIOGENESIS IN THE SUBVENTRICULAR ZONE INDUCED BY EPIDERMAL GROWTH FACTOR

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The ErbB receptor family mediates a plethora of effects in a wide array of tissue types and contexts. Signaling through the ErbB receptors is often altered in cancerous tumors. Increased ErbB signaling, mainly ErbB1 (EGFR) and ErbB2, can lead to increased proliferation and invasiveness of tumor cells. There is also evidence suggesting a role of ErbB1 in angiogenesis, characterized by expression of ErbB1 in endothelial cells. We have previously described that intracerebroventricular infusion of epidermal growth factor (EGF) induces dysplasia in hyperproliferative polyps in the subventricular zone (SVZ). Interestingly, blood vessels develop in about 30% of the polyps after continuous EGF infusion for 14 days. Structurally, the newly formed vessels are of a disorganized and glomeruloid appearance. Sometimes as bundles sprouting from a single SVZ blood vessel, and other times as a highly vascularized ventricle wall. These vessels are to a large extent covered by pericytes strongly expressing NG2. However, NG2 is also expressed by other cell types such as polydendroglia and oligodendrocyte progenitors. We found cells expressing NG2 throughout the brain; however, pericytes strongly expressing NG2 were restricted to the dysplastic areas. This indicates that neoangiogenesis is not a general phenomenon in the EGF infused SVZ but specifically induced in hyperproliferative areas. The putatively angiogenic area showed multi-luminal vessels and signs of immaturity based on ultrastructural characteristics, such as a thickened endothelial cell layer. In addition, these vessels exhibited a dysfunctional blood-brain barrier, demonstrated by albumin extravasation using Evans blue. Using an antibody against phosphorylated EGFR we found phospho-EGFR-positive endothelial cells exclusively in the angiogenic foci. These results suggest angiogenic properties of EGF, specific to hyperproliferative areas in the SVZ.

Poster Board Number: T-1099

MUSASHI1 POST-TRANSCRIPTIONALLY REGULATES THE GENE EXPRESSION IN NEURAL STEM/PROGENITOR CELLS AND GLIOMA.

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Musashi is an evolutionarily conserved family of RNA-binding proteins that is expressed in the nervous system. In *Drosophila*, this protein plays an essential role in regulating the asymmetric cell division of sensory organ precursor cells through the translational regulation of target mRNA (Nakamura et al., Neuron 1994; Okabe et al., Nature 2001). Its mammalian homologues, Musashi1 and Musashi2, are RNA-binding proteins that are expressed in fetal and adult neural stem/progenitor cells (NS/PCs). Previously, our group

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reported that the transcript of m-numb was identified as target of Musashi1, and were repressed the translation by Musashi1 through inhibition of mutual interaction of two translational initiation proteins, PABP and eIF4G (Imai et al., Mol Cell. Biol., 2001; Kawahara et al., J. Cell Biol. 2008). We and other group independently demonstrated that Musashi1 and Musashi2 activate the Notch signaling pathway by translational repression of the mRNA for the Numb protein, a negative regulator of the Notch-signaling pathway (Imai et al., Mol Cell. Biol. 2001; Ito et al., Nature 2010; Kharas et al., Nat. Med. 2010). Other Musashi1-target transcripts, p21waf1, doublecortin, and APC were identified by our group and other groups (Battelli et al., Mol. Cell. Neurosci. 2006; Horisawa et al., FEBS Lett. 2009; Spears and Neufeld, J. Biol. Chem. 2011). However, entire Musashi1-target mRNAs in self-renewing neural stem cells had remained to be elucidated. For the purpose of revealing whole of Musashi1-RNA networks in NS/PCs, we purified endogenous Musashi1-RNA complexes in NS/PCs by immunoprecipitation, and identified Musashi1-associateing mRNAs by using gene expression microarray method. Interestingly, the result showed that many mRNAs of tumor-related genes, cell cycle-regulating genes and differentiation-regulating genes were concentrated by Musashi1-specific immuno-purification. In order to know whether Musashi1 regulates self-renewal ability through the regulation of the Musashi1-associating mRNAs in NS/PCs and tumor cells, we focused some tumor-related genes among them. siRNA ablation studies against musashi1 were performed by using low times-passaged glioblastomas, established glioblastoma cell lines, and medulloblastoma cell line, which form 'gliomasphere' in the stem cell culture condition with both EGF and FGF2. The decrease of Musashi1 in tumor cells led to increased expression of the Musashi1-target genes including Numb and PTEN. Furthermore, our results showed the reduction of post-transcriptional regulation by Musashi1 impaired self-renewing activity and cell survival competence of glioma cells through the deactivation of Notch and PI3K-Akt signaling pathways. Taken together, our observation suggested that Musashi protein plays an important role in the self-renewing cells in the tumor as well as NS/PCs.

Poster Board Number: T-1100

REGULATION OF NEURAL STEM CELL SELF-RENEWAL AND DIFFERENTIATION

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Wnt signaling plays a critical role in regulating self-renewal and differentiation of neural stem cells. We previously reported that a Wnt receptor, Ryk intracellular domain (ICD) translocates into the nucleus and regulates GABAergic neuronal differentiation. However, it remains to be elucidated how Ryk ICD moves to the nucleus and regulate neuronal differentiation. We have recently identified the nuclear protein Smek as a Ryk ICD interacting protein. Smek expression is upregulated during neuronal differentiation and is sufficient to drive the nuclear localization of Ryk ICD. In addition, Smek overexpression increased neuronal differentiation in Ryk-dependent manner. To further examine roles of Smek in neurogenesis *in vivo*, we have generated Smek double knock out mice. We demonstrated that Smek is required for GABAergic neuronal differentiation and directly regulate the expression of Dlx transcription factors which are essential for GABAergic neuronal differentiation. These data suggest that Smek might be a key regulator of Wnt-Ryk signaling in GABAergic neuronal differentiation of developing mice brain.

Epithelial Cells (Not Skin)

Poster Board Number: T-1101

IDENTIFICATION OF MELANOCYTE STEM CELLS IN ECCRINE GLANDS AS A POTENTIAL SOURCE OF ACRAL MELANOMA

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Human cutaneous melanoma is a highly aggressive cancer that is resistant to traditional cancer treatments including both chemo- and radio-therapy1-3. Acral melanoma is the most prevalent subtype of melanoma in the non-Caucasian population. The preferential proliferation of early acral melanoma cells along and around epidermal eccrine ducts (EG), a reliable early diagnostic sign of melanoma with a 99% specificity4, has indicated a close association between early acral melanoma *in situ* and EGs. However, neither the presence of melanocytic cells in the eccrine sweat glands nor the precise origin of these melanoma cells is known as in most cancers. Here, we report the identification of melanocyte stem cells (MelSC) in mouse and human acral skin. We identified unpigmented melanoblasts residing in the secretory portion (SP) of EGs, using lineage-tagged H2B-GFP reporter mice. These melanoblasts are normally kept in an immature, slow-cycling state but are able to self-renew, indicating that this population possesses adult stem cell features in the niche area. In response to stress, including ionizing irradiation, they not only renew themselves but also provide amplifying and differentiating progeny that migrates upward toward the epidermis where they mature into melanin pigment-producing melanocytes. In addition, we found that a similar population expressing melanocyte lineage marker MART1 reside in the secretory portion of the EGs in human acral skin. Analysis of early acral melanoma *in situ* revealed that distribution of MART1+ proliferating melanoma cells are not localized only within the epidermis but accompanied by their contiguous distribution starting from the SP of a particular EG through the connecting eccrine ducts (ED) in early melanoma lesions. Thus, we propose that the EG-MelSCs are the potential source of human acral melanoma that produces melanoma initiating cells during their constitutive renewal.

Poster Board Number: T-1102

GENERATION OF AN *IN VITRO* MODEL OF CYSTIC FIBROSIS

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Cystic Fibrosis (CF) is an autosomal recessive inherited chronic lung disease and with over 70,000 patients worldwide it is associated with severe disability and a short life expectancy. Current animal models of CF poorly simulate clinical lung disease and a lack of primary lung tissues has limited the rate of research progress. With this in mind we are using patient derived iPSC to generate a reproducible *in vitro* model of CF with potential for a matched positive control using homologous recombination techniques to correct the identified mutation. Provirus free iPSC were generated from fibroblasts isolated from a skin biopsy obtained from a CF

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organ donor. iPSC were generated using a CRE excisable six factor polycistronic lentiviral reprogramming cassette that we developed and colonies were picked at day 14 of reprogramming. The provirus was excised using CRE-RNA and excision was confirmed by absence of a PCR product for the lentiviral backbone and the SOX-2/KLF4 junction in the reprogramming plasmid. Both iPSC and provirus iPSC were fully characterized for stem cell pluripotency including endogenous gene expression, immunofluorescent imaging of colonies, EB formation, teratoma formation of all three germ layers and routine karyotype analysis. The genotype of the patient was confirmed to be delta 508 homozygous and the mutation was retained across all cell types generated; fibroblasts, iPSC and provirus free iPSC. Using both CF iPSC and control fibroblast derived iPSC a differentiation protocol was developed consisting of a differentiation to definitive endoderm (SOX17, GATA6 positive) and plating of the cells first in a liquid-liquid and then air-liquid interface in transwell inserts. A timecourse of RNA analysis by qPCR indicates an increase in definitive endoderm markers GATA6, SOX17 and NKX2.1, which is important in early lung development and differentiation of the distal lung compartments. As differentiation progresses generation of cells expressing FOXJ1 and acetylated α tubulin is indicative of a push towards ciliated cells and the expression of pulmonary surfactant proteins B, C and D indicates alveolar cell differentiation. Cells also formed tight junctions indicated by ZO-1 and epithelial Cadherin staining and strongly expressed cytokeratin 18 and to a lesser extent cytokeratins 14 and 19. Furthermore, there is a distinct change in the expression of CFTR in differentiated cells incubated for 24 hours at 30°C with notable increase in cell surface expression observed at the lower temperature. In conclusion we have generated the first (to our knowledge) functional patient specific model of cystic fibrosis with the capacity for a congenic positive control via homologous recombination and correction of the mutant gene.

Poster Board Number: T-1103

BIOENGINEERING OF HUMAN CORNEAL EPITHELIAL STEM/PROGENITOR CELLS BY MODULATING THE WNT SIGNALING PATHWAY

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Wingless (Wnt) signaling plays a critical role in the regulation and maintenance of stem cells. This study investigates the Wnt signaling pathway in human corneal epithelial stem/progenitor cells. Differential gene profiling of the human limbus, conjunctiva and cornea revealed that there were 146 transcripts preferentially expressed in the limbus where the putative corneal epithelial stem cells are located. Wnt signaling pathway was among the notable biological processes, and Wnt6 and Fz7 preferential expression in the limbus was confirmed by qRT-PCR and immnohistochemistry. When the primary human limbal epithelial cell culture was supplemented with Wnt6 using the Wnt6 overexpressing mouse 3T3 feeder cells, the expression of putative stem cell markers, ABCG2 and corneal maturation marker, keratin 12 expression had a 3 fold decrease. On the other hand, loss of the stem/progenitor phenotype in culture correlated with a lower expression level of Fz7. In addition, knockdown of Fz7 expression using shRNA in the primary human limbal epithelial cells led to the loss of stem/progenitor phenotype. Colony forming efficiency was significantly decreased. In summary, our data indicated that Wnt signaling pathway regulates the differentiation of the limbal stem/progenitor cells and Fz7 might be responsible for transducing the Wnt signaling. Modulating the Wnt signaling could increase the efficiency of limbal stem/progenitor cell expansion *in vitro*.

Poster Board Number: T-1104

ELUCIDATING THE ROLE OF ACTIVATED NICHE SIGNALS IN REGULATING HUMAN LIMBAL STEM CELLS PROLIFERATION AND MAINTENANCE

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Tissue-specific stem cells play a key role in facilitating the maintenance of self-renewing tissues and repair after injury. Evidence suggested that corneal epithelial stem cells reside within the basal layer of the limbus, the narrow zone between the cornea and the bulbar conjunctiva. In response to injury cues, dormant LESCs are efficiently activated and produce numerous progenitors and mature cells. Diseases that affect the limbal epithelial stem cells(LESCs), such as chemical burn, thermal burn, and Steven-Johnson syndrome can leads to condition called limbal stem cell deficiency, characterized by conjunctival epithelial ingrowth, vascularization, and chronic inflammation, and eventually can lead to vision loss. The molecular mechanisms by which limbal niche signals affect LESCs fate decisions during normal tissue homeostasis and during injury state are largely unknown. In this study, we found that TGF-β, the main during corneal wound repair, strongly promoted BMP antagonists, noggin and gremlin, and VEGF expression in the underlying limbal stromal cells. When LESCs were culture 3T3 cells overexpressing BMP antagonists, LESCs exhibited significant higher colony forming capability than when cultured on unmodified 3T3 cells. Cells from colonies grown on 3T3-noggin however, displayed more differentiated phenotypes and reduced the ability to sustain serial passages suggested the loss of self-renewal capability. Gene expression analysis also showed an activation epithelial-mesenchymal transition (EMT) transcription program. Importantly, when small molecule modulators of TGF-β signaling were applied to the culture system, LESCs can be serial passage for more than 2 months (>10 passages) while still exhibited high level of P63 expression and retained their ability to generate holoclones. Modulation of niche signals could also reverse the phenotypic changes of LESCs grown on 3T3-noggin and enable long-term serial culture. Taken together, our results underline the potential of therapeutic strategies targeting TGF-β/ BMP signaling in corneal injuries with/or without LESCS transplantation.

Poster Board Number: T-1106

REPROGRAMING OF HUMAN AMNIOTIC EPITHELIAL CELLS BY OCT4 OVEREXPRESSION

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Objectives; Oct4 is supposed to be a key factor of cell reprogramming, as iPS cells have been established by introduced the combination of Oct4, Sox2, Klf4, and c-Myc or Oct4, Sox2, Nanog, and Lin28. Here, we examined the possibility of cell reprogramming by overexpressing Oct4 using electroporation. Methods; Plasmid vector harboring Oct4 gene downstream of CMV-promoter was introduced into immortalized human amniotic epithelial cells (iHAEs) using electroporation. Each gene expression was detected by RT-PCR, quantitative RT-PCR, and protein expression was detected by immunocytochemistry and flowcytometric analysis. Results; Although the expression of Oct4 gene and protein was increased immediately by introducing Oct4 gene, they were decreased chronically and acceleratingly since two days after the introduction. The expression of stemness marker gene Rex1 was increased two days after the introduction and SSEA4 positive cells increased 5-10%

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four days after the introduction. SSEA1, differentiation marker, positive cells decreased 10-20% eight days after the introduction. Conclusion; The expression of Rex1 and SSEA4 was increased and the expression of SSEA1 was decreased with Oct4 overexpression. It is suggested that Oct4 induces cell reprogramming.

Poster Board Number: T-1107

INHIBITION OF PROTEIN NITROSYLATION ENHANCES LIVER REGENERATION AFTER TOXIC INJURY

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Acetaminophen (APAP) liver toxicity is the most common cause of fulminant liver failure. Currently, there are no treatments to enhance recovery from toxic injury. Signaling pathways that modulate embryonic liver development may help to elucidate conserved mechanisms that effect liver regeneration. In order to identify novel regulators of liver development *in vivo*, we performed a chemical genetic screen in zebrafish embryos, which revealed that nitric oxide (NO) signaling modulated liver development. The NO donor S-nitroso-glutathione (GSNO) caused increased liver size at 72 hpf, assessed by in situ hybridization for liver fatty acid binding protein (lfabp) and quantified by FACS of GFP-labeled hepatocytes. In contrast, NO inhibition through N-nitro-L-arginine methyl ester (L-NAME) caused smaller livers. GSNO and L-NAME altered expression of the hepatoblast marker hhex, indicating that NO signaling affects the liver progenitor pool during liver development. NO mediated its effects not via the classical pathway of cGMP signaling, but through protein nitrosylation: while modulation of cGMP had no impact on liver size, knockdown or chemical inhibition of the negative regulator of protein nitrosylation, GSNO reductase (GSNOR), resulted in larger livers. In order to determine whether NO signaling also impacted recovery after liver injury, we employed previously validated larval and adult zebrafish models of APAP liver toxicity: zebrafish exposed to APAP develop hepatocyte necrosis, as demonstrated by elevated alanine aminotransferase levels and histology, and death. Treatment of APAP-exposed fish with a novel GSNOR inhibitor (N6547, N30 Pharmaceuticals) significantly prevented the rise in alanine aminotransferase, improved hepatocyte necrosis and enhanced cell proliferation, as demonstrated by BrdU incorporation. Furthermore, GSNOR inhibition improved survival by more than 50% following APAP exposure. Patients with APAP liver toxicity may not immediately present after drug ingestion, reducing the efficacy of the only available clinical antidote, N-acetylcysteine (NAC): GSNOR inhibition acted synergistically with NAC after delayed treatment up to 18 hours after APAP exposure, thereby expanding the therapeutic window to improve outcome. In order to demonstrate a conserved role of GSNOR in mammalian liver injury, we treated wild-type and GSNOR knockout mice with sublethal doses of APAP: wild-type mice exhibited an increase in alanine aminotransferase levels at 6 and 24 hrs, whereas GSNOR-/- mice had significantly lower values. Furthermore, wild-type mice developed characteristic pericentral necrosis following APAP exposure, while GSNOR-/- had minimal injury. These data demonstrate that NO signaling, acting via nitrosylation, affects liver progenitors to ensure optimal liver development and regeneration following APAP induced liver injury. We propose that NO can act as a rapid signaling molecule to sense injury and initiate the regenerative response. GSNOR is an excellent candidate for novel therapeutic approaches to improve the regeneration and outcome in APAP liver failure.

Poster Board Number: T-1108

PLURIPOTENT TRANSCRIPTION FACTOR OCT4 PROMOTES CELL MIGRATION IN ENDOMETRIOSIS

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BACKGROUND: Endometriosis is the growth of endometrial tissues eutopically and/or ectopically which present high cell migration ability. Pluripotent transcription factor OCT4 has been associated endometriosis. However, the role of OCT4 in endometriosis still remains largely unknown. This study aims to examine the expression profile of pluripotent transcription factor OCT4 in human ectopic endometriosis progression and its role in migration of endometrium cells. METHODS AND RESULTS: We analyzed the gene and protein expression level of pluripotent transcription factor OCT4 and NANOG in patient tissues (n=110) by quantitative real-time RT-PCR and immunohistochemical staining. While comparison with human endometrium (n=2) and myoma (n=4), the hyperplasia (n=37) and ectopic endometriosis tissues (n=67, 19 for adenomyosis and 48 for chocolate cyst) showed a significantly increasing of OCT4 and NANOG expression in mRNA level. The OCT4 protein was both detected in stromal and luminal epithelial cells of adenomyosis- and chocolate cyst tissues. The OCT4 expression level in endometriosis tissues is positively correlated with the gene expressions which associated with cell migration, such as Twist, Snail, Slug, and Vimentin. Overexpression of OCT4 protein in human endometrium carcinoma cell lines RL95-2 and HEC1A (both in low OCT4 expression level) significantly increased the expression level of migration-associated genes (Twist, Snail, Slug, N-cadherin, and Vimentin) and proteins (N-cadherin and Vimentin); and promoted the migration of endometrium cells which was evidenced by wound healing- and transwell assay. Immunocytochemical staining combined with confocal images further demonstrated that OCT4 affects actin filament distribution and the migratory morphology of human endometrium cells. CONCLUSIONS: OCT4 expression in endometrium cells showed a high impact on ectopic endometriosis progression and significantly increased the migration ability of endometrium cells. Findings in this study may provide a potential early diagnosis biomarker and part of the molecular mechanism in the progression of human ectopic endometriosis and ovarian cancers.

Poster Board Number: T-1109

P18 DELETION IMPROVES STEM CELL SELF-RENEWAL, ORGAN HOMEOSTASIS, AND LIFESPAN OF TELOMERE DYSFUNCTIONAL MICE WITHOUT AFFECTING DNA DAMAGE CHECKPOINTS

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Telomere shortening limits the lifespan of primary cells and tissues by induction of p53 dependent checkpoints limiting the self-renewal of stem cells and tissue homeostasis. The cell cycle inhibitor p18 regulates G1 cell cycle transition and self-renewal of hematopoietic stem cells but its role in DNA damage induced aging has not been explored. Here we show that the deletion of p18 leads to improvements in stem cell self-renewal in the intestinal epithelium and in the hematopoietic system of telomerase knockout mice with dysfunctional telomeres. These improvements in stem cell main-

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tenance result in improved organ homeostasis and an elongated lifespan of the mice. The study shows that telomere dysfunction does not induce an upregulation of p18 and the deletion of p18 does not ameliorate telomere dysfunction or the induction of DNA damage checkpoints. Together, these results provide the first experimental evidence that improvement of stem cell self-renewal can prolong organ homeostasis and lifespan in the context of activated DNA damage checkpoints.

Poster Board Number: T-1110

THE ROLE OF SLIT/ROBO SIGNALING IN MOUSE MAMMARY STEM CELL SELF-RENEWAL AND PROGENITOR DIFFERENTIATION

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In the breast, SLIT/ROBO signaling is important for normal development and it also acts as a tumor suppressor pathway. Our data suggest that secreted SLIT proteins, signaling through ROBO receptors, are crucial components of the mammary extracellular environment, and may be important for regulating division of mammary stem cells (MaSCs) and differentiation of their progenitors. Double knockout of Slit2 and Slit3 in mammary gland serial transplants results in enhanced longevity. In wild-type (WT) mammary glands, this procedure can be performed serially for 5-6 generations before no further outgrowths are obtained. In Slit2-/-;Slit3-/- tissue, however, four independently-derived lines of tissue have been serially transplanted up to 13 generations, suggesting that loss of SLIT/ROBO signaling leads to a delay in MaSC senescence. Conversely, chronic treatment of the Lin-CD29hiCD24+ (DP) population, which is enriched for MaSCs, with SLIT2 *in vitro* decreases self-renewal, as assessed by passaging ability, in comparison with untreated DPs. Whereas SLIT2 treated DPs can be passaged 3-4 times before undergoing senescence or terminal differentiation, untreated DPs can be passaged 7-8 times. Together, these data confirm the importance of SLITs as regulators of MaSC self-renewal. SLIT/ROBO signaling also appears to act independently of its MaSC self-renewal effects to regulate progenitor activity and differentiation. *In vitro*, luminal progenitors (Lin-CD29loCD24+CD61+) derived from Slit2-/-;Slit3-/- mammary glands give rise to larger colonies, indicating that these progenitors proliferate more to give rise to excess daughter cells. Treatment of WT luminal progenitors with SLITs does not affect colony size, but does increase colony number, suggesting that SLITs may help to keep luminal progenitors from differentiating into mature luminal cells. These data suggest that SLIT/ROBO signaling may influence mammary gland development by regulating progenitor activity and differentiation. Current studies are focused on examining whether SLIT/ROBO signaling regulates MaSC and progenitor cells through candidate downstream pathways such as Wnt and Notch signaling. In summary, our research shows that SLIT/ROBO signaling is an important regulator of the mammary epithelial cell hierarchy by promoting non-renewal of MaSCs as well as proliferation and differentiation of their progenitor offspring.

Liver Cells

Poster Board Number: T-1111

BI-POTENT HEPATIC PROGENITORS DERIVED FROM HUMAN PLURIPOTENT STEM CELLS FOR DRUG TESTING

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Primary human hepatocytes are a valuable tool for biomedical research, the pharmaceutical industry and therapeutic applications. They are becoming increasingly utilized in drug development to evaluate human specific drug properties such as metabolic fate, drug-drug interactions and drug toxicity. However, the demand for primary human hepatocytes far exceeds the available supply and the quality of primary hepatocytes is highly variable. Human embryonic stem cells (hESCs) could in principle provide a renewable source of human hepatocytes. However, the derivation methods described so far are inefficient and do not yield pure populations of functional mature hepatocytes. Our preliminary study showed that TW6 hESCs differentiate into hepatocytes via a progenitor stage in which cells were capable of extensive growth. By expanding these progenitors in culture and inducing hepatic differentiation, a pure population of hepatocytes could be generated with high efficiency and shorter time course. Thus the focus of this study was to develop a platform for derivation and expansion of hepatic progenitors from hESCs. To achieve this, hESCs were differentiated into cells expressing hepatic progenitor markers at day 13, followed by expansion in a serum-free expansion medium for two weeks. The expanded cells displayed proliferative ability as indicated by Ki67 expression, exhibited high nucleus-to-cytoplasm ratio, and expressed hepatic progenitor markers such as AFP and EpCAM. These cells were passaged at confluency using mechanical dissection. Furthermore, the progenitors could be induced to differentiate into albumin-positive hepatocytes or CK7-positive cholangiocytes, exhibiting bi-potent differentiation potential. The hepatic progenitor cells can be utilized as a renewable source of hepatocytes and may potentially be useful for cell therapies, bioartificial livers, hepatitis C virus infection cell model and drug testing.

Poster Board Number: T-1112

GENERATION OF CGMP-COMPATIBLE FUNCTIONAL HEPATOCYTES FROM A CLINICAL-GRADE HUMAN EMBRYONIC STEM CELL LINE

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Human embryonic stem cells (hESC) hold great potential for use in regenerative medicine and drug development. However, growing and maintaining hESC on mouse feeder layers with the use of animal products hinders their clinical applications. Recently hESC have been developed as clinical-grade cell lines by BioTime. In this study, we have attempted to generate cGMP-compatible functional hepatocytes employing a clinical-grade hESC line and feeder-free (FF) and xeno-free conditions. Line ESI-035 (ESI35) was recovered, expanded and maintained on either mouse feeder cells, human feeder cells, or Matrigel, then differentiated into hepatocytes. The ESI35-derived hepatocytes (ESI35-Hep) were characterized by the expression of liver-specific proteins and functions, as well as by

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metabolic profiling of the drug bufuralol (BF). Under our modified conditions (no animal products), ESI35 cells grown on mouse or human feeders or on Matrigel were initially induced to contain a high percentage of definitive endoderm (DE) cells as assessed by the expression of SOX17 and CXCR4 at levels between 95% and 98%, as determined by flow cytometry (FC), and by showing a uniform morphology when immunostained. Employing our xeno-free differentiation protocol and FDA certified fetal bovine serum, the DE cells were successfully differentiated into hepatocytes with 91-98% of cells positive for albumin (ALB) and α1-antitrypsin after 3-4 weeks of differentiation, as determined by FC and immnu-ohistochemistry. The cellular uptake and excretion of Indocyanine Green was observed in our ESI35-Heps, indicating a functional biotransforming system, and the cells also showed glycogen accumulation. ELISA results demonstrated that ESI35-Hep grown on mouse feeder cells (ESI35-hep/MF) secreted 5.3 ± 0.8 µg ALB into the medium per million cells over 24h after differentiation, ESI35-Hep grown on human feeder cells (ESI35-Hep/HF) secreted 5.0 ± 0.4 µg ALB, and ESI35-Hep grown on Matrigel (ESI35-Hep/FF) secreted 2.7 ± 0.28 µg ALB. To further assess the biotransformation system, we employed ultraperformance liquid chromatography-tandem mass spectrometry technology for BF metabolic profiling and metabolism. We found the same 3 secondary metabolites of BF from oxidation, dehydrogenation, ketone formation, or potential methylation in phase I, and the same 2 secondary metabolites of BF from glucuonidation and conjugation of glucose in phase II, in all of the ESI35-Heps when compared to freshly isolated human primary hepatocytes (hPH). Interestingly, the metabolite of di-oxidation was only detected in ESI35-Hep/HF and hPH, and importantly, the levels of all secondary metabolites in ESI35-Hep/HF were significantly higher than those in ESI35-Hep/MF and ESI35-Hep/FF, suggesting that human feeder cells may promote and enhance the differentiation of hESC. Thus it appears that our ESI35-Heps/HF have developed full metabolic function. In conclusion, these results demonstrate that clinical-grade hESC can be effectively differentiated to hepatocyte-like cells employing cGMP-compatible culture conditions. This represents an important initial step in the use of differentiated hESC for cell-based therapeutics.

Poster Board Number: T-1113

ESTABLISHMENT OF STABLE HEPATOCYTE PROGENITOR CELL LINE FROM HUMAN EMBRYONIC STEM CELLS

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It is well known that hepatocytes are important for assessment tools for toxicological research and pharmaceutical development, however, acquisition of human hepatocytes is not easy. Human embryonic stem cell (hESC)-derived hepatic progenitor offer a potential supply for functional hepatocytes. The goal of this study is to establish stable hESC-derived hepatic progenitor cell lines. The first stage involves the formation of definitive endoderm by activin A and WNT3a. The second stage we induced to proliferation and differentiation of hepatic progenitor by KSR and DMSO. In this stage, we introduced pEGFP-α-fetoprotein (AFP) into hepatocyte progenitor by viral transfection. We isolated pEGFP-AFP positive hepatocyte progenitor cells (AFP-HPCs). We characterized hepatic lineage the marker gene expression in the isolated AFP-HPCs. In addition we terminally differentiated matured hepatocyte-like cells by using AFP-HPCs (the third stage). The hESC-derived stable hepatocyte progenitor cell lines provide a valuable source for hepatocyte by simple manipulation, and may be useful as *in vitro* system for toxicity screening in drug discovery.

Poster Board Number: T-1114

DEVELOPMENT OF EFFICIENT HEPATIC DIFFERENTIATION METHODS FROM HUMAN IPSCS AND ESCS BY USING LOW-MOLECULAR WEIGHT COMPOUNDS

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Hepatocytes are useful cells in a variety of fields, such as liver cell therapy, drug screening, and toxicity testing. Primary human hepatocytes are often used for these purposes. However, since these primary cultured cells easily lose their metabolic functions, a substitute for primary hepatocytes is required. Human induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs) are potential sources for unlimited supply of hepatocytes. There are several reports describing the directed induction of iPSCs and ESCs into hepatoblasts, which are progenitors of hepatocytes, and hepatocyte-like cells by the combinational treatment of growth factors. However, the induction efficiency is still low and the generated cells show immature phonotypes, including lack of key detoxification enzymes Cyp3a4 and glucose-6-phosphatase. In this study, we examined efficient methods to induce human iPSCs into hepatocytes by using low-molecular weight compounds. To identify low-molecular weight compounds that can induce hepatoblasts into hepatocytes, we performed high-throughput screening(HTS). A human iPSC line, 201B6, was induced into hepatoblasts with a previously-described differentiation protocol. Generated hepatoblasts were then treated with chemical compounds. After 8-12 days of culture with the tested compounds, cells were immunostained with ALBUMIN, a representative marker for hepatocytes. We evaluated the inducing ability of the compounds by analyzing the induction rate of iPSCs-derived hepatocytes. Out of 1,120 compounds examined, we have found two candidate compounds (compounds X and Y) which induced iPSCs-derived hepatoblasts into ALBUMIN$^+$ cells more efficiently than control stimulus (HGF and Oncostatin M). Next, we investigated the concentration dependency of these compounds and found that the effective concentration was 20-20,000 nM in both compounds. In conclusion, we have identified candidate compounds that would enable the efficient and low-cost differentiation from human iPSCs into hepatocytes. We are now examining the mechanisms of action of the two compounds and physiological functions of the generated ALBUMIN$^+$ cells.

Poster Board Number: T-1115

CHARACTERIZATION OF HUMAN-INDUCED HEPATIC STEM CELLS AND THEIR DIFFERENTIATION BY A ONE-STEP PROTOCOL

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Human hepatocytes are useful for *in vitro* testing in drug discovery, but the lack of available donor hepatocytes is a major obstacle for application. Some sets of genes can induce pluripotent stem (iPS) cells from postnatal tissues of an individual patient. Hepatocyte-like cells generated from iPS cells might also be useful in non-clinical testing or regenerative medicine. The *in vitro* hepatic differentiation

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of human iPS cells required a complicated procedure, such as the addition of several growth factors by a three-step protocol. Otherwise, hepatocyte-like cells reprogrammed directly from other cells did not expand further with their hepatic function. If self-renewing hepatic stem cells could be provided, they would have an advantage in practical use. Gene transfer of OCT3/4, SOX2, and KLF4 could also induce human hepatic stem (iHS) cells from the skin or gastric tissues of an adult patient. Microarray analysis revealed that three clones of iHS cells markedly expressed many hepatic genes (more than 50 genes); in addition, these cells expressed embyonic stem (ES) cell-enriched genes (more than 20 genes). Quantitative RT-PCR analysis also confirmed that iHS cells expressed hepatic genes (ALB, AFP, SERPINA1, and TTR); in addition, these cells expressed ES cell-specific genes (OCT3/4, SOX2, NANOG, and ZFP42) at an equivalent level. Co-expression of hepatic and stem cell markers was confirmed by immunocytochemistry. The resulting iHS cells were self-renewed without chromosome abnormalities *in vitro* for more than one year. They were similar to human ES cells and iPS cells in morphology. Such expandable cells would have an advantage in application. After 2 weeks of culture without the addition of growth factors for differentiation, the long-term self-renewed iHS cells could differentiate into hepatocyte-like cells that expressed various hepatic markers 10^3 to10^5 times more than those of iHS cells and produced hepatic proteins in a culture supernatant at mg/dL. Altogether, these results suggested that iHS cells not only provide new insight into stem cell research but also have an advantage for application.

Poster Board Number: T-1116

COMPARATIVE STUDY OF TRANSPLANTATION OF HEPATOCYTES AT VARIOUS DIFFERENTIATION STAGES INTO MICE WITH LETHAL LIVER DAMAGE

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Hepatocyte transplantation utilizing induced pluripotent stem cells (iPSCs) or embryonic stem cells (ESCs) has been expected to provide an alternative to liver transplantation. However, it remains uncertain precisely which cell type is the best suited for cell transplantation. In particular, it is unclear whether mature hepatocytes, which have sufficient liver function, or immature hepatic progenitor cells, which have a higher proliferative capacity, will provide a better outcome. The main objective of this study was to investigate the therapeutic efficacy of the transplantation of hepatocytes at various differentiation stages. We utilized transgenic mice that expressed diphtheria toxin (DT) receptors under the control of an albumin enhancer/promoter. ESC-derived endodermal cells, fetal hepatocytes and adult hepatocytes were transplanted into these mice with experimentally-induced lethal acute liver injury caused by DT administration. The transplanted cells were marked by enhanced green fluorescent protein. We evaluated their effects on survival. At 35 days after transplantation, the survival rate of the adult hepatocyte-transplanted group (8/20; 40.0%) was significantly improved in comparison to that of the sham-operated group (2/25, 8%), the fetal hepatocyte-transplanted group (1/20, 5%) and the ESC-derived endodermal cell-transplanted group (0/21, 0%). The adult hepatocytes proliferated in the recipient livers and replaced a large part of their parenchyma. The transplantation of adult hepatocytes for acute liver failure significantly improved the survival rate in comparison to that of transplantation of immature cells, thus suggesting that ESCs and iPSCs should be differentiated into mature hepatocytes before cell transplantation for acute liver failure.

Poster Board Number: T-1117

INDUCTION OF FUNCTIONAL HEPATOCYTES-LIKE CELLS FROM MOUSE MESENCHYMAL STEM CELLS BY FOXA2 FOR LIVER DEVELOPMENT

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Induced hepatocyte-like cells have multiple hepatocyte-specific features and aids in reconstituting damaged hepatic tissues after transplantation. To differentiate mouse Mesenchymal stem cells into functional hepatic cells using FOXA2 - a master regulator of liver-specific gene expression for liver development. FOXA2 - a hepatocyte nuclear factor 3β (HNF3β) gene was transfected into mMSCs followed by G418 selection. The cells were differentiated and these cells were confirmed by immunocytochemistry, RT-PCR, PAS and western blot. Functional hepatocyte-like cells show typical epithelial morphology, express hepatic genes and acquire hepatocyte functions. Notably, these cells expressed the markers of mature hepatocytes, including albumin, tyrosine aminotransferase, α1-antitrypsin, Cyp7A1, and hepatic transcription factors such as hepatocyte nuclear factors 4α and 6. Furthermore, these cells exhibited hepatic functions *in vitro*, including glycogen storage and cytochrome activity. We have developed a robust and efficient method to differentiate mesenchymal stem cells into hepatic like cells, which exhibits characteristics of mouse hepatocytes. Our approach would facilitate the development of hepatocytes for liver engineering and regenerative medicine.

Poster Board Number: T-1118

POLYCOMB GROUP PROTEIN RING1B REGULATES PROLIFERATION AND DIFFERENTIATION OF MOUSE HEPATIC STEM/PROGENITOR CELL BY REPRESSING CYCLIN-DEPENDENT KINASE INHIBITORS

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Self-renewal of somatic stem cells is thought to be satisfied that the integrated control of multiple gene expression relating to cell proliferation and differentiation. Recent studies have revealed that the histone modifiers, including the Polycomb group (PcG) proteins, play important roles in stem cell self-renewal. Here, we address PcG regulation of mouse hepatic stem/progenitor cell self-renewal through inactivation of Ring1B that is essential for gene silencing by PcG proteins with monoubiquitin E3 ligase activity towards histone 2A. Functional analyses of PcG protein Ring1B in hepatic stem/progenitor cell were conducted by Ring1B conditional knock-out (cKO) mice. In Ring1B-cKO fetal mice, we found that the liver size was lessened significantly by Ring1B depletion on early stage of liver development that hepatic stem/progenitor cell would expand their population. In contrast, there was no decline in liver size in Ring1B-cKO mice with lately depletion. Immunohistochemical staining analysis revealed that numbers of both alpha-fetoprotein+ undifferentiated and BrdU+ cells were decreased in Ring1B-cKO mice liver. When performed functional analysis of Ring1B in c-kit- CD49f+ CD29+ CD45- Ter119- hepatic stem/progenitor cells from Ring1B-cKO mouse with single cell-base colony assay *in vitro*, the

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significantly suppression of clonal expansion and cellular senescence was found by Ring1B depletion. And decreased numbers of albumin/cytokeratin 7 double positive cells were detected in the colonies generated from Ring1B depleted hepatic stem/progenitor cells. Microarray and ChIP-on-chip analysis were performed to narrow the Ring1B downstream candidate gene related hepatic stem/progenitor self-renewal regulation. It showed cyclin-dependent kinase inhibitor gene not only Cdkn2a but also Cdkn1a expression, increased as the liver development progresses, were derepressed in Ring1B-cKO liver cells. We performed functional analysis of Cdkn1a- or Cdkn2a-Ring1B double KO mouse to evaluate directly involvement of these genes in Ring1B cKO mice hepatic stem/progenitor cells. Cdkn1a- or Cdkn2a-Ring1B double KO mice did not affect the undifferentiated cell growth or rescue liver mass size, while depression both Cdkn1a and Ckdn2a expression in hepatic stem/progenitor cells from Ring1B KO mice rescued their colony formation capacity compared to Ring1B cKO mice These results suggest that Ring1B thus affect regulation of proliferation in hepatic stem/progenitor cell self-renewal by repressing cell cycle related genes both Cdkn1a and Cdkn2a.

Poster Board Number: T-1119

DECREASED LEVELS OF HEPATOCYTE GROWTH FACTOR IMPROVE LIVER CELL ENGRAFTMENT IN A GENETICALLY ENGINEERED MOUSE MODEL

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Background & Introduction: Developing methods to enhancing cell engraftment is important for cell/stem cell therapy. We have generated a mouse line with narrowed sinusoidal diameters, which is associated with decreased levels of hepatocyte growth factor (HGF). In the current study, we aimed at testing whether cell engraftment can be improved in an HGF-insufficient background. Method: Primary hepatocytes from R26R-GR mice (a double-fluorescent-reporter knockin mouse line that could be used as a reporter system for investigation of cell therapy conditioning) were isolated by a two-step collagenase perfusion. Approximately 1 million of viable hepatocytes were infused into wild-type (WT) or HGF-insufficient mice through the splenic pulp. At different time points, frozen liver sections were analyzed and the absolute number of transplanted cells was scored using a fluorescence microscope. Result: The data showed that the nuclei of tissue sections of R26R-GR mice were fluorescently labeled as expected. Cultured primary hepatocytes were also fluorescently labeled in nuclei, indicating that the primary cells derived from R26R-GR mice could be a useful tracing tool for cell/stem cell therapy. Equivalent number of the primary hepatocytes was transplanted into WT or HGF-insufficient mice, and then the efficiency of cell engraftment was analyzed at 3, 24, and 48 hours after transplantation. The data showed that the efficiency of cell engraftment was significantly greater in HGF-insufficient mice than that in WT mice at all the time points ($n = 3$/group; cells/30 fields; WT vs. HGF-insufficient; 3 hours: 15.00 ± 4.81 vs. 28.60 ± 2.26, $p < 0.001$; 24 hours: 22.20 ± 4.24 vs. 37.60 ± 9.05, $p = 0.001$; 48 hours: 22.17 ± 4.40 vs. 40.80 ± 7.23, $p < 0.001$). Conclusion: We demonstrated that decreased levels of HGF could enhance cell engraftment, and that the R26R-GR mouse line could be a good donor of cell sources for the research on cell/stem cell therapy. We suggest that this strategy, antibody pretreatment against HGF for instance, may be applicable to improve cell/stem cell therapy in the near future.

Poster Board Number: T-1120

PROTECTIVE EFFECT OF ADIPOSE TISSUE-DERIVED STEM CELLS (ADSCS) FOR ACUTE LIVER FAILURE IN MICE

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Background: Adipose tissue-derived stem cells (ADSCs) have been shown to proliferate and differentiate into multiple cell lineages. ADSCs is emerging as novel therapeutic option for regenerative medicine. Recently, several publications have reported that ADSCs have therapeutic potential in different diseases of animal models and clinical application. Here, we found the alternative source of ADSCs - omentum adispose tissue, which contain abundant stem cell population. The aim of this study is to investigate the therapeutic potential of omentum ADSCs on acute liver failure (ALF) induced by acetaminophen (APAP) in mice. Methods: We demonstrated the therapeutic efficiency of omentum-derived ADSCs transplantation for ALF mouse model induced by APAP. In this study we plan to identify if the omentum-derived ADSCs can be a new source of donor cells. Firstly, we will isolate and in vitro culture the ADSCs from mouse omentum tissue. The characteristics and differentiation ability of omentum-derived ADSCs will analyze by flow cytometry for MSC marker (CD29, CD31, CD34, CD44, CD90, and CD105 etc), immunostaining for multiple cell lineage (hepatocyte, osteoblast, and adipocyte). The protection capacity of omentum-derived ADSCs was evaluated on primary in vitro cultured hepatocytes treated with APAP and demonstrated by MTT assay, reactive oxygen species (ROS) detection, glutathione content measure, and antioxidant enzyme activity (catalase, superoxide dismutase, and glutathione peroxidase) expression. Secondly, we will study the *in vivo* therapeutic efficiency of these omentum-derived ADSCs in ALF mice. We will transplant intrasplenically the omentum-derived ADSCs (1million cells/mice) into ALF mice. The serum samples will be taken at hour 8, 12, 24, and 48 to measure the liver function (GOT, GPT, and t-bill). We will detect the profile of antioxidant enzyme activity on liver tissue after omentum-derived ADSCs transplantation in ALF mice and observe the survival benefit. Moreover, we will assess the difference of JNK pathway expression after ADSCs transplantation. Results: Omentum-derived ADSCs showed morphological similarity to the MSC, and expressed the MSC characteristics and properties of differentiation. Omentum-derived ADSCs could protect the hepatocytes to against the APAP toxicity by decreasing the ROS production, enhancing the intercellular GSH content, and increasing the antioxidant enzyme activity. The omentum-derived ADSCs could rescure the individual survival and improve the liver function in ALF mice. The profiles of antioxidant reveal that omentum-derived ADSCs could increase the activity of antioxidant enzyme. Furthermore, JNK-mediate pathway can be inhibited in recipient mice with omentum-derived ADSCs transplantation. Conclusion: These studies demonstrated the omentum-derived ADSCs are a novel cell source for cell-based therapy in ALF. And the omentum-derived ADSCs could against the hepatotoxicity and protect the hepatocytes by reducing the ROS production, increasing the antioxidant enzyme capacity via the inhibition of the JNK-mediated pathway.

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Poster Board Number: T-1121

ADULT HEPATIC PROGENITOR CELL AND INJURED LIVER REGENERATION IN FISH MODELS

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Background: Chronic liver disease encompasses a large number of conditions having different etiological factors, progressing from fibrosis to cirrhosis and possibly liver failure or hepatocellular cancer. Chronic liver disease is a leading cause of mortality in China and the United States, responsible for the deaths of more than 200,000 Chinese and 25,000 Americans each year. Although there are about 17,000 people require liver transplants in the USA, only 5,000 cadaveric liver transplants are estimated to be available per year. Adult stem cells are an attractive alternative to meet this huge clinical demand, but the current understanding of stem cell biology in adult liver is limited. By using zebrafish and medaka models, this project aims to investigate the presence of liver progenitor cell in adult and their cellular and molecular characteristics. Materials and Methods: A transgenic zebrafish line was used in this study. The mature hepatocytes in this line are labeled with GFP with the expression driven by a gene promoter from the liver fatty acid binding protein (FABP), which is specifically expressed in mature hepatocytes. After liver profusion, Flow Activated Cell Sorting was conducted based on their expression of GFP and Patched (Ptc), the Hedgehog receptor. Each isolated fraction of cells was: 1) analyzed by Q-RT-PCR to detect expression of other stem cell markers; and 2) tested for their potential to differentiate into hepatocyte and/or biliary epithelial cell in vitro and *in vivo* systems. Results: In an adult liver from a FABP-GFP transgenic zebrafish (6 months old), 70-85% of cells were sorted as GFP positive (GFP+) and expressed albumin, consistent with mature hepatocytes with cell size of 10-15µm. However, about 10-15% of the total liver cell populations were GFP negative (GFP-, non-hepatocyte) with cell size of 5-10 µm. There were 0.4 - 0.5% of the isolated liver cells with both GFP negative and Ptc positive (GFP-/Ptc+). The cells in the GFP-/Ptc+ fraction also has enriched expression of Ptc and Aldh2 mRNA, 30 fold and 23 fold, respectively, when compared to GFP positive cells. The GFP negative cells can be cultured in vitro on collagen IV and fibonectin coated plate and induced to differentiate into GFP positive hepatocyte under HGF/FGF cocktail incubation for 5 days. One week after transplantation of the GFP- cells into wild type see-through Medaka fish pretreated with liver toxin, tunucamycin, resulted in cell lineages of biliary epithelial cells and hepatocytes both express GFP. As few as ten GFP-/Ptc+ cells can regenerate and rescue over 90% chemical-induced injury liver within 14 days. Conclusion: Conditions for isolating a cell fraction which we believed consisted of liver progenitor cells from adult zebrafish fish have been established. Further studies will be conducted to investigate the localization of these cell types in adult zebrafish liver and their detailed molecular features. These studies provide a starting point for further characterization of unique cell types in adult liver that may be important in liver regeneration and even in hepatocarcinogenesis.

Poster Board Number: T-1122

MEK ACTIVITY REGULATES STEM/PROGENITOR POTENTIATL OF FETAL HEPATOBLASTS THROUGH INDUCTION OF CELL CYCLE ARREST

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Introduction: Fetal livers contain several types of cells, such as hepatoblasts (stem/progenitor cells which have bi-potency to differentiate into hepatocytes and cholangiocytes), hematopoietic cells, mesenchymal cells, and endothelial cells. Knockout mouse studies of mesenchymal-marker transcription factors (Hlx and Lhx2) revealed that mesenchymal cells is important for liver development. We previously established a new culture system which mimic the interaction between early-fetal (embryonic day 9.5-10.5 in mouse) hepatoblasts and mesenchymal cells; expansion of hepatoblasts is significantly induced by the co-culture with mouse embryonic fibroblasts (MEF). However, the molecular mechanism maintaining stemness of fetal hepatoblasts remains largely unknown. In this study, we analyzed the signal pathway regulating long-term proliferation of fetal hepatoblasts in vitro using several signal molecule specific inhibitors. Methods: Fetal hepatoblasts were purified using specific cell-surface antibodies (Dlk and CD133). Dlk+CD133+ hepatoblasts were sorted and cultured at a low density (200 cells per 12-well dish) with MEF as feeder cells for 6 days. Then, these cells were trypsinized and prepped onto new feeder cells. Colony formation derived from sorted cells were analyzed using immunocytochemistry. Signal molecule-specific inhibitors (a Rock inhibitor [Y-27632], a GSK-3 beta inhibitor [CHIR99021], a MEK inhibitor [PD325901], a TGF beta inhibitor [A83-01]) were added into culture media for the analyses of cell signal pathways. Expression of cell cycle associated molecules were analyzed using real-time PCR. Results: Individual fetal hepatoblasts could large colonies derived from single cells in the co-culture with feeder cells for 6 days (the 1st culture). After the passage into new feeder cells (2nd culture), these cells could barely large colonies, suggesting that this culture system could not maintain long-term proliferation. In contrast, when a MEK inhibitor was added into the culture, fetal hepatoblasts could form large colonies which express have many Ki-67-positive cells in the 2nd culture. Expression of both p15ckdn2b and p16/19cdkn2a was induced in this culture system, whereas a MEK inhibitor significantly suppressed expression of these cdk inhibitors. Fetal hepatoblasts derived from p16/19cdkn2a knockout mice proliferated for a long time without a MEK inhibitor. These expanded cells highly expressed p15ckdn2b, suggesting that long-term proliferation of fetal hepatoblasts in vitro was suppressed by expression of p16/19cdkn2a, which was induced by a MEK-ERK signal pathway. In addition, overexpression of p16 or p19 in fetal hepatoblasts derived from p16/19cdkn2a knockout mice decreased colony formation. Using the transplantation assay, fetal hepatoblasts expanded in the presence of a MEK inhibitor could engraft and proliferate in the injured-recipient livers. Conclusion: We established a new culture system which can expand fetal hepatoblasts for a long time. The activation of MEK-ERK pathway *in vitro* caused the induction of cdk inhibitor p16/19cdkn2a. A MEK inhibitor suppressed this signal activation and maintained the long-term proliferative potential of fetal hepatoblasts in this culture. This culture system could be useful for cell therapies using *in vitro* expansion of a few hepatic stem/progenitor cells.

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Poster Board Number: T-1123

STEM CELL BASED GENE THERAPY: A NOVEL APPROACH FOR THE TREATMENT OF ALPHA-1-ANTITRYPSIN DEFICIENCY

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The human SERPINA1 gene encodes for the secretory protein alpha 1-antitrypsin (A1AT) which inhibits a wide variety of proteases in the serum by covalent binding. In humans a missense point mutation (E342K) was described which leads to the expression of the PiZ isoform of A1AT. Homozygosity for PiZ (PiZZ) leads to severe alpha 1-antitrypsin deficiency. In this case the serum concentration of A1AT is reduced by 90%, as the protein is not able to fold correctly, but polymerizes and is retained in the endoplasmic reticulum of hepatocytes. Patients show an increased breakdown of elastin by elastase in the lung and, therefore, are suffering from lung emphysema or chronic obstructive pulmonary disease. Moreover, the accumulation of misfolded protein in the liver causes liver function disorders and liver cirrhosis. We investigate new strategies for the treatment of the liver disease in severe A1AT deficiency using a transgenic mouse model overexpressing the human PiZ protein. From these mice we generated induced pluripotent stem (iPS) cells using lentiviral vectors encoding the three transcription factors Oct4, Sox2 and Klf4. After reprogramming the PiZ-iPS were transduced with a second lentiviral vector encoding for a miR30-styled shRNA, which is directed specifically against the point-mutated form of the human SERPINA1 transgene. We also cloned an eGFP reporter directly in front of the knockdown shRNA to track its expression through all stages of differentiation. These gene-corrected PiZ-iPS cells were then subjected to several hepatic differentiation protocols to evaluate the shRNA-mediated rescue of the diseased phenotype. Moreover, gene-corrected PiZ-iPS were injected into blastocysts for characterization of the SERPINA1 knockdown *in vivo*. Next, we transduced iPS cells generated from a human PiZZ individual with liver disease with our knockdown construct. These gene-corrected cells were also differentiated along the hepatic lineage and showed significantly reduced expression of SERPINA1 when compared to scramble-shRNA transduced cells. We have successfully evaluated a novel treatment for the liver disease in A1AT deficiency employing stem cell based gene therapy and we show evidence for a significant reduction in the expression of a harmful gene in a mouse model for a human disease and in cells from a human patient.

Poster Board Number: T-1124

MMP-9 PROMOTES THE TRAFFICKING OF TRANSPLANTED BONE MARROW CELLS THAT ATTENUATES HEPATIC FIBROSIS IN CHRONIC CCL4 LIVER INJURY

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Background: Recent clinical trials have shown that transplanted bone marrow (BM) cells migrate into injured liver and attenuate hepatic fibrosis. However the mechanism by which BM cells mobilize into damaged liver or how the transplanted BM cells work *in vivo* are still under investigation. Purpose: Using MMP-9 (-/-) (KO) BM cells, we tried to investigate the role of MMP-9 in the trafficking of BM cells and in the contribution to the regression of hepatic fibrosis. Methods: 1) In vitro, BM cells were isolated from MMP-9 KO and MMP-9 (+/+)(WT) mice, and Matrigel invasion activity was assessed by transwell chambers. 2). *In vivo*, chronic liver injury was induced to C57BL/6 female mice by injection of CCl4 twice weekly for 8 weeks. At 4th week, mice were transplanted with BM cells (1×107/mouse) isolated from male WT or KO mice which were genetically positive with EGFP. Non-transplanted (non-TP) control mice were injected with normal saline alone. The liver specimens were examined by real time qPCR, collagen content assay, and immunohistochemical studies. Results: 1) Matrigel invasion assay showed WT-BM cells significantly migrated into the lower chamber (2.5 fold) compared with KO-BM cells. 2) Mice transplanted with WT-BM cells (WT-TP group) displayed approximately 2.0 fold of EGFP (+) cells along the fibrotic tissue and around central veins compared to those transplanted with KO-BM cells (KO-TP group). Real-time qPCR also showed EGFP and sex-related Y chromosome expression was significantly higher in WT-TP group than KO-TP group. 3) Liver fibrosis, assessed by Sirius red staining, was prominent in non-TP control group, whereas KO-TP and WT-TP groups revealed 50% and 70% decrease of fibrotic area respectively, examined by VH analyzer. Collagen content assay was coincident with Sirius red staining. The mRNA expressions of alpha1(I) collagen in both WT-TP and KO-TP groups were significantly lower compared with that of non-TP group. However expressions of MMP-13 and MMP-9 in WT-TP group were significantly higher than those in KO-TP group. 4) Immunofluorescent microscopy showed most EGFP (+) cells were co-stained with F4/80, a marker of macrophage, and most of them co-expressed MMP-9 and MMP-13 in TP groups. Immunoelectron microscopy demonstrated EGFP(+) cells showed the morphology containing many lysosomes in the enlarged cytoplasm, suggesting the characteristic features of macrophages. Conclusion: MMP-9 has a crucial role in the BM cell migration into injured liver. Transplanted BM cells, mostly differentiated into macrophages, contribute to regression of hepatic fibrosis via down-regulation of type I collagen and up-regulation of MMP-9 and MMP-13.

Poster Board Number: T-1125

PREDICTION OF STEM CELL ASSOCIATED MICRORNA IN HEPATOCELLULAR CARCINOMA

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Background Cancer stem cells (CSCs) are associated with drug-resistance and poor patient prognosis. The microRNAs (miRNA) involved in CSC of hepatocellular carcinoma (HCC) are not well revealed. We hypothesized that CSC shares the common miRNA profiles of embryonic stem cells (ESCs). The aim of this study is to find out potential significant miRNAs in CSC hepatocarcinogenesis by comparing the miRNA profiling of HCC to that of ESCs. Method A total of 84 patients with HCC received primary resection between 2002 and 2006 were enrolled in this study. HCC(T) and nearby non-tumor liver (N) tissues were sampled for miRNA Illumina BeadArray (total 1145 microRNAs) and validated by Real-Time PCR analysis. Ten samples from living donor (D) livers were used as control. Candidate miRNAs were selected by using paired-T or T test with FDR correction and p-value <0.05 and TN (or TD, ND) ratio >2 or <0.5. Selected miRNAs were further reviewed to identify the very miRNAs significantly involved in ESC miRNA profiling

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based on current literature. Clinicopathologic characteristics and survivals were analyzed. Result Three of 15 differently expressed miRNAs were sieved out to be ESC-related in TN comparison (up: miR 96; down: miR 199a-5p, miR 200a), 11 of 96 in TD comparison (up: miR 372, miR 34a, miR 221; down: miR199a-5p, miR 200a, miR 130a, miR 154*, miR 494, miR 19b, miR 19a, miR 301a), and 7 of 57 in ND comparison (up: miR 34a, miR 221; down: miR 154*, miR 494, miR 19b, miR 19a, miR 301a). In TN comparison stratified on the hepatitis viral status, 5 of 27 miRNAs selected in the HBV (up: miR 130b, miR 96, miR 18a, miR 17-5p:9.1; down: miR 199a-5p), 3 of 28 in the HCV (down: miR 199a-5p, miR 214, miR 154), and 3 of 8 in the non-HBV/HCV groups (down: miR 200b, miR 200a, miR 429). Lower expression of miR 200b*, but not miR 200, predicted poorer patient survival (P = 0.026, log-rank test). Selected miRNAs were associated with pluripotency (miR 372, miR 494, miR 154*, miR 199a), lineage differentiation or development (miR 154, miR 34a), control of epithelial-mesenchymal transition (EMT) (miR 200a, miR 200b, miR 429), oncogenic function (self-renewal) (miR 18a, miR 19a), and regulation of general cell physiology (miR 16, miR 19b, miR 130, miR 130b). Higher percentage (37.5%) of dysregulation of miRNAs in the non-HBV/HCV group was associated with control of EMT than the HBV or HCV group. MiRNAs associated with pluripotency (miR 494, miR 154*, miR 19a) were found down-regulated in non-tumor liver tissues N compared to healthy donor liver tissues D. Conclusion Selected miRNAs potentially significantly associated with CSC in HCC were reported. Further mechanism of CSC hepatocarcinogensis is going to be investigated.

Poster Board Number: T-1126

THE LYMPH NODE AS AN ECTOPIC TRANSPLANTATION SITE FOR MULTIPLE EPITHELIAL TISSUES

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Cell-based therapy has been viewed as a promising alternative to organ transplantation. However, for some patients, orthotopic cell-based therapy directed at a diseased organ may not be feasible for many reasons, ranging from a possible lack of an appropriate environment in cirrhotic and fibrotic liver during end-stage disease to the lack of a thymus in complete DiGeorge Syndrome. Consequently, a critical component of cell-based therapy for these patients is to establish an optimal in vivo site for cell and tissue transplantation to restore organ functions. Here, we address the challenge of determining a suitable location and test the hypothesis that transplantation of three distinct normal epithelial cell types directly into the lymph nodes (LNs) of mice would engraft and demonstrate ectopic organ functions in vivo. Previously, our lab has shown that primary hepatocytes injected intraperitoneally into a mouse model of tyrosinemia type I (Fumarylacetoacetate hydrolase knockout mouse, Fah-/- mouse) migrate and colonize the host abdominal lymphatics and restore hepatic function. In this report, we demonstrate that directly injected hepatocytes can engraft in the jejunal LN and respond to generic liver injury (hepatectomy) by proliferating. We also show that hepatocytes injected directly into a single jejunal or extra-abdominal (popliteal or axillary) LN will generate an ectopic hepatic mass and rescue Fah-/- mice from lethal liver failure. The LN was transformed into a hepatic organoid composed of characteristically cuboidal hepatocytes organized in what resembled normal liver architecture but with the absence of a biliary system. Taken together, our results demonstrate that direct injection of hepatocytes into a single LN generates ectopic liver tissue in an animal model of liver disease. Furthermore, this result suggests that LNs provide a beneficial environment for ectopic transplantation of other cell types. Using a similar approach, we asked if de novo thymus function could be generated in LNs of athymic mice. Thymuses were harvested from newborn GFP mice, minced and injected directly into the jejunal LN of athymic nude mice. We found that the ectopic thymuses in the LN contained recipient double positive thymocytes as well as single positive CD4 and CD8 T cells, indicating a selective mechanism of T-cell commitment and maturation. Moreover the de novo T cell-mediated immune system rejected xenogeneic tumor growth in the majority of the LN treated nude mice. Together, these data support the concept of using the LN as a site for thymic transplant to generate an ectopic thymus. Finally, pancreatic islets were harvested from GFP mice and transplanted into the jejunal LN of streptozotocin-induced diabetic mice. The ectopic islets transplanted in a single jejunal LN of the mice engrafted and secreted insulin to decrease glucose levels. We provide the first report describing the use of a LN as a site for cellular transplant. By directly injecting the LN with hepatocytes, thymuses, or pancreatic islets, we demonstrate engraftment of the donor cells and subsequent organ function. This approach will be beneficial to the field of regenerative medicine and provides a new concept to use the LN as an in vivo bioreactor in which to regenerate functional organs.

Poster Board Number: T-1127

PROPAGATION OF ADULT STEM/PROGENITOR CELLS IN A SERUM–FREE THREE–DIMENSIONAL CULTURE SYSTEM

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Recent progress has demonstrated adult liver stem/progenitor cells as potential sources for generating transplantable liver cells. However, the great variability in methods utilizing to isolate liver stem/progenitor cells is a considerable challenge for clinical applications. A serum-free three-dimensional culture system was established in this study for selection and propagation of adult liver stem/progenitor cells. We demonstrated that, when adult liver cells were grown on polyvinyl alcohol (PVA) coated glassware, adult liver stem/progenitor cells can form spheres. These sphere cells expressed a panel of stem-cell markers including CD133, EpCAM, CD49f, AFP, CK19 and Oct-4 and had the potency to differentiate into hepatocytes and cholangiocytes when co-culturing with fetal liver cells. Moreover, transplantation of GFP-labeled sphere cells to the liver of mice treated with 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) could replenish damaged hepatocytes. Moreover, overexpression of HBx in isolated sphere cells resulted in the formation of tumors with some characteristics of hepatocellular carcinoma and cholangiocarcinoma upon intrasplenic injection into immunodeficient mice. These data provide evidences for the stem cell-like capacity of these PVA-cultured sphere-forming cells. In conclusion, we develop a simple, rapid and label-free method for prospectively isolating hepatic stem/progenitor cells from the adult mouse liver which will facilitate their application in repairing injured liver.

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Poster Board Number: T-1128

GENOMIC DNA METHYLATION AS A POTENTIAL MARKER OF STEM CELL DURING HEPATIC CELL DIFFERENTIATION

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[Purpose] Technological improvements of stem cell differentiation into hepatocytes have increased the momentum of their application to drug metabolism and toxicity testing. Therefore, to develop the assessment of these cells is an important issue. Currently, evaluation is performed in the differentiated hepatocytes by the cell morphology, enzyme activity, and expression of differentiation markers. However, differentiated hepatocytes lost their proliferation ability. Thus, if one can assess the differentiation ability of the cells prior to hepatocyte, it might help to accelerate cell production and banking. HepaRG cells, a hepatocyte progenitor, have the ability to differentiate towards hepatocyte-like and biliary epithelial-like cells at confluence. We reported that genome wide DNA methylation did not change significantly during the HepaRG cell differentiation (62th JSSX annual meeting). This observation suggested that evaluation of genomic DNA methylation in the progenitor cell might be useful for the assessment of differentiation potential. To investigate the validity of genomic DNA methylation assessment system, we performed analyses of genome wide genomic DNA methylation and gene expression. [Methods] Genomic DNA and total RNA from human primary hepatocytes (three donors), HepaRG cells, and HepG2 cells were used for genome wide analyses of genomic DNA methylation and gene expression. Genomic DNA methylation and Gene expression analyses were performed with Human Methylation 450 BeadChip Arrays (Illumina) and GeneChip Human Genome U133Av2 Array (Affimetrix), respectively. [Results and Discussion] Correlation between frequencies of DNA methylation in 89794 CpG sites on CpG Island in 13029 gene regions and the gene expression levels were compared among primary human hepatocytes, HepaRG cells, and HepG2 cells. As the result, frequencies of DNA methylation in 1751 CpG sites in 582 gene regions showed negative correlation with their gene expression levels. The cluster analysis of these genes indicated the direct suppression of gene expression by DNA methylation. Evaluation of the gene set as a marker for the cell characterization is in progress. [Acknowledgement] Part of this research was supported by the INSERM/Japan Society for the Promotion of Science (JSPS) cooperation program.

Poster Board Number: T-1129

SMALL MOLECULE LIBRARY SCREENS FOR HEPATIC MATURATION OF INDUCED PLURIPOTENT STEM CELL DERIVED HEPATOCYTES *IN VITRO*

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Human hepatic models such as primary human hepatocytes, HepaRG and HepG2 cells have been used to evaluate components of liver function *in vitro*. However, none of these models maintain the full hepatocyte (HC) functionality found *in vivo*. Pluripotent stem cells (PSCs) have the potential to provide a robust and accessible *in vitro* model of human hepatic function. However, despite development of methodologies to differentiate PSCs into HCs that exhibit features of mature, adult HCs, PSC derived HCs retain some features of immature, fetal HCs, in particular with respect to their drug metabolizing cytochrome P450 activity. Thus, in attempts to develop PSC derived HCs with more adult-like functionality that may be useful as a tool during early drug discovery activities, we performed small molecule screens to identify compounds capable of modulating the PSC HC phenotype. Compounds from three unique small molecule libraries were screened for the ability to induce further maturation of human induced pluripotent stem cell derived HCs (iCell Hepatocytes) from Cellular Dynamics International. These include a 299 compound kinase inhibitor library, a 94 compound epigenetic modulator library, and a 296 compound library of small molecules known to be active in one or more cell based assays previously employed elsewhere in the drug discovery program at Roche. iCell Hepatocytes were plated in a 96-well format. Cells were treated with the compound libraries at a concentration of 5 uM at 24 hrs and 72 hrs after plating. In addition, in some instances different cell culture conditions were examined such as incubating the cells at low (6.5%) oxygen concentrations. Total RNA was harvested 24 hrs after the final compound treatment. Experimental gene expression was evaluated in triplicate by Fluidigm microfluidic qPCR using 32 probes chosen based on their differential expression observed during a previously performed microarray analysis of RNA purified from untreated iCell Hepatocytes, human fetal and human adult HCs. Furthermore, the screening probes were picked in order to span major HC functional activities including cytochrome P450, transporters, bile acid synthesis, and key hepatic proteins and transcription factors. The Ct values of each probe set examined were first normalized to the expression values of housekeeping gene controls. Next, the compound treated values were compared to the corresponding gene expression level observed in vehicle (DMSO) control treated samples. Top compound hits were chosen based on a compound's ability to alter the gene expression in a manner predicted to increase cellular maturity, for instance an increase of adult specific markers or a decrease in fetal specific markers. The top 30 positive compounds from the primary screen were then tested at multiple doses during a secondary screen. In total, greater than 300,000 individual endpoints were measured for the primary and secondary screen analysis. Currently the top 5 compounds that exhibited reproducible gene expression changes during the secondary screens are being further studied with the goal of identifying the mechanisms of action and functional consequences.

Pancreatic Cells

Poster Board Number: T-1131

INVESTIGATION OF THE HUMAN EMBRYONIC STEM CELL DERIVED PANCREATIC PROGENITOR CELL ALTERATION IN *IN VIVO* ENVIRONMENT

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Development of a cell therapy for diabetes would be greatly aided by a renewable supply of human beta-cells. One approach to overcoming the problem of insufficient supply is to generate islets from proliferative stem cell populations such as human embryonic stem cells. By establishing hESC expansion and banking methods and a

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suspension-based differentiation system, hES cell were aggregated into clusters in rotational suspension culture, followed by differentiation for two weeks with a 4-stage protocol to produce pancreatic progenitors. We had previously shown these pancreatic progenitors further differentiate into mature pancreatic cells, including glucose-responsive insulin-secreting cells, when implanted into immune compromised animals. To further assess the *in vivo* graft development mice were sacrificed at various time points post-engraftment, ranging from 1 to 22 weeks, for assessment of mRNA gene expression and histological analysis. We observed temporal changes in gene expression consistent with pancreatic progenitor differentiation to the endocrine lineage as well as subsequent maturation of endocrine cell phenotypes. Observation of grafts by histological analysis confirmed these findings and suggested a conversion of poly-hormonal endocrine cells to glucagon-expressing phenotypes, as previously shown in other work. Investigation of cell proliferation by Ki67 staining indicated proliferative cell number is relatively low and does not change dramatically during this time course of post-engraftment analysis. Interestingly, both exocrine and duct cell protein expression was observed after the endocrine cell maturation, and this was consistent with the gene expression data. The majority of the dynamic changes in cell phenotypes and gene expression were detectable before robust glucose-responsive C-peptide secretion was observed in the serum of these animals at 10wk post-engraftment. These data serve to further elucidate the time course and mechanisms by which implanted pancreatic progenitor cells differentiate and mature to become functional glucose-responsive cells.

Poster Board Number: T-1132

MESENCHYMAL TO EPITHELIAL TRANSITION IS REQUIRED FOR REPROGRAMMING HUMAN EXOCRINE ENRICHED PANCREATIC CELLS TOWARDS BETA-LIKE CELLS

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Due to the shortage of islets available for transplantation, the *in vitro* generation of functional beta cells is currently accepted as one of the most promising sources of tissue for transplantation in the treatment of type 1 diabetes. The aim of this study was to reprogramme redundant human exocrine tissue towards islet cells, to be used as a patient specific "top-up" supply of pancreatic islets. Human Exocrine Enriched Pancreatic Cells (EEPCs) were obtained from the exocrine part of the pancreas which is usually discarded during the islet isolation procedure and propagated *in vitro*. These cells attach to tissue culture dishes and assume a mesenchymal morphology in culture, with abundant expression of vimentin and loss of exocrine markers, such as amylase and CK19. In order to instigate the reprogramming of the cultured EEPCs, the exogenous expression of four pancreatic transcription factors (TFs) was induced with the addition of Ad-Ngn3, Ad-Pdx1, Ad-MafA and Ad-Pax4 to these cultures, in combination with Betacellulin, Nicotinamide and Exendin-4 (GFs). The effect of the TFs and GFs was enhanced by pre-incubating the cells with 5-aza-deoxycytidine and Sodium Butyrate, which inhibit DNA methylation and deacetylation, respectively. After this treatment, high levels of insulin and glucagon were detected in the reprogrammed EEPCs by RT-qPCR, immunocytochemistry and in the culture medium by ELISA. These cells were cultured in Serum Free Medium, which led the cells to undergo a Mesenchymal to Epithelial Transition (MET) and this was crucial for the efficiency of reprogramming. Furthermore, it was also found that the GFs were capable of enhancing MET, by inducing the expression of the epithelial marker E-cadherin and the down regulation of the mesenchymal marker vimentin. Therefore, MET appears to have an important role in the reprogramming of adult pancreatic cells towards the endocrine cell fate.
These studies were complemented with the transplantation of the reprogrammed endocrine cells into the kidney capsule of NOD/SCID mice. Immunohistochemical analysis of the graft revealed that the cells maintained a cluster-like morphology under the kidney capsule, being positive for the endocrine hormones glucagon, insulin and somatostatin, with a morphology similar to the structure of a pancreatic islet.

Poster Board Number: T-1133

COMPARATIVE EXPRESSION OF MICRORNAS AND MRNAS DURING DIFFERENTIATION OF PANCREATIC ISLET-LIKE CELL CLUSTERS FROM HUMAN EMBRYONIC AND ADIPOSE-DERIVED MESENCHYMAL STEM CELLS

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Type 1 diabetes is an autoimmune destruction of pancreatic islet beta cell disease, so it is important to find new sources of the islet beta cells to replace the damaged cells. Human embryonic stem (hES) cells have the potential to provide an unlimited supply of differentiated islet-like cells for tissue replacement. However, this method needs an immune-suppression to prevent islet rejection. Autologous islet-like cells differentiated from adult adipose-derived mesenchymal stem cells (MSC) are now considered to be a good source for cell therapy of type 1 diabetes. The hES-T3 cells with normal female karyotype and MSC from normal adult female were separately induced using different protocols to generate the pancreatic islet-like cell clusters, which expressed pancreatic islet cell-specific markers of insulin, glucagon and somatostatin. The expression profiles of microRNAs and mRNAs from the pancreatic islet-like cell clusters differentiated from the two sources of hES-T3 and MSC were analyzed and compared. The pancreatic islet-like cell clusters differentiated from the hES-T3 cells were found to exhibit very high expression of microRNAs miR-186, miR-199a and miR-339, which down-regulated the expression of LIN28, PRDM1, CALB1, GCNT2, RBM47, PLEKHH1, RBPMS2 and PAK6. The expressions of microRNAs and mRNAs from the islet-like cell clusters differentiated from the MSC are being determined, and will be compared with those of the islet-like cell clusters differentiated from the hES-T3 cells. The microRNAs and their target genes are very likely to play important regulatory roles in the development of pancreas and/or differentiation of islet cells, and they may be manipulated to increase the proportion of beta cells and insulin synthesis in the differentiated pancreatic islet-like cell clusters for cell-therapy of type I diabetics.

Poster Board Number: T-1134

INDUCTION OF PANCREATIC CELLS FROM HUMAN IPS CELLS IN A SERUM-FREE MONOLAYER CONDITION

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Remarkable advance in stem cell biology has made it possible to apply for regenerative therapy. Especially, by finding of the method

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for obtaining iPS cells, we can have differentiated cells from patients to possess pluripotency and can re-induced appropriate tissues and organs. This strategy may have applicability to the new therapeutic treatment for diabetes. Insulin-dependent diabetes is occurred by loss of pancreatic beta cells, resulting in shortage of insulin and elevation of blood glucose level. To the remedy for this malady, transplantation of pancreatic beta cells is effective. To date, serum-containing medium and feeder cells are ordinarily used to maintain pluripotency and differentiate cells, but this condition is afraid to contaminate unknown factors, resulting in both instability of experimental condition and doubt about the safety of the therapy. Here, we report the induction of pancreatic cells from human iPS cells in serum and feeder cell free condition. Based on the previous reports, we adopted modified strategy. In this method, we sequentially added various cytokines such as Activin, Wnt, FGF, RA, EGF, GLP and Insulin. By using this method, a group of cells with patch-like shape could be observed. Immunohistological analysis revealed that many of these cells were stained with anti-C-peptide antibody, suggesting that insulin-secreted cells could be induced. Furthermore, glucagon positive cells could be also seen. Together with these results, we expect the functional pancreatic islet-like structure could be differentiated from human iPS cells. However, induction efficiency was not so high. To overcome this problem, further improvements have to need. For example, to increase the ratio of appropriately induced beta cell, we are now optimizing the timing and duration of treating with cytokines. In this report, we will also discuss this problem.

Poster Board Number: T-1135

SINGLE ADULT MURINE DUCTAL PANCREATIC STEM CELLS SELF-RENEW AND BUILD MULTILINEAGE CYSTIC STRUCTURES *IN VITRO*

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It remains unclear whether adult pancreatic ducts harbor stem cells. Here, we tested whether purified adult murine pancreatic ductal cells, which express CD133 and Sox9, may be induced to differentiate and self-renew in a novel three-dimensional clonogenic assay in the absence of a mesenchymal niche. CD133+Sox9/EGFP+ cells from dissociated adult murine pancreata were sorted using a fluorescent activated cell sorter and differentiated in vitro in a semi-solid medium containing methylcellulose, Matrigel, and growth factors (J Vis Exp, 2011, PMID: 22143165). The single cell-derived colonies were characterized by microfluidic RT-PCR, immunostaining and electron microscopy. We found that about 17% of sorted, single murine CD133+Sox9/EGFP+ ductal cells built cystic structures containing cells that resemble ductal, acinar and endocrine cells. Immunostaining and electron microscopy analyses demonstrated that the differentiated ductal-like cells displayed the correct polarity. Microfluidic RT-PCR analysis of individual colonies indicated that more than 70% expressed markers for ductal, endocrine and acinar lineages, suggesting that the majority of the colony-initiating cells are multipotential. Furthermore, single cells from approximately 80% of primary colonies formed secondary cystic colonies, suggesting self-renewal activities, and R-Spondin 1, a Wnt signaling agonist, increased the self-renewal capacity of serially passaged clonal cells. Finally, partial duct ligation in vivo increased the ratio of non-colony-forming to colony-forming ductal cells, demonstrating a dynamic response of ductal progenitors to injury. To our knowledge this is the first report of the ability of single adult ductal pancreatic stem cells to self-renew and differentiate into multiple lineages in vitro. The clonogenic culture system described will simplify the study of pancreatic stem cell biology and advance the field of regenerative medicine in treatment of diabetes.

Poster Board Number: T-1136

DIFFERENTIATION OF MURINE EMBRYONIC STEM CELLS AND OF MURINE SKIN-DERIVED MESENCHYMAL STEM CELLS INTO INSULIN-PRODUCING CELLS

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Isolated pancreatic islet transplantation is an alternative treatment indicated for hyperlabile type 1 Diabetes mellitus (DM1), however, a large number of good quality islets is required to achieve DM1 reversion. New cell source alternatives have been investigated, the most promising being insulin producing cells (IPCs) differentiated from stem cells. Some reports show that murine embryonic stem cells (mESCs) are able to form islet-like structures, but insulin production is insufficient to achieve normoglycemia in diabetic mice, the same occuring with adult stem cells from different tissues. Therefore, in order to revert Diabetes, there is great demand for an adequate protocol to achieve sufficient amounts of insulin-producing cells from differentiated stem cells . Aiming at an adequate protocol to differentiate stem cells into insulin-producing cells, we subjected mESCs to embryoid bodies formation, followed by introduction of a set of endoderm differentiation inducers, and treatment with beta-cells differentiation factors. Early on the mESCs differentiation, we noticed some epithelial-like cells emerging from the embryoid bodies culture. After 10 days of differentiation, we detected the expression of INS2, NGN3, ISL1 and GLUT2 by qRT-PCR. At the end of our protocol, we noticed the formation of islet-like clusters, which were positive for the insulin-specific dithizone staining and expressed INS2, PDX1, NGN3, ISL1 and GLUT2 transcripts. In addition, murine skin-derived mesenchymal stem cells (mS-MSCs) were induced to differentiate with both the same factors used for mESC differentiation and conditioned medium collected from differentiating mESCs. Although mS-MSCs subjected to differentiation with the factors were positively stained by dithizone, the levels of insulin and other transcripts related to β-cell differentiation were undetectable. This may be explained by the low amount of differentiated mS-MSC producing insulin and the inability of the factors used in our protocol to induce mS-MSC complete differentiation into insulin producing cells. On the other hand, conditioned medium obtained from differentiating mESCs was able to induce low levels of the insulin gene transcription. This suggests that factors secreted by mESCs during their differentiation process contribute to their own differentiation and may also contribute to differentiation of other stem cell types. Therefore, we were able to develop a new protocol, based on pancreatic organogenesis, which seems to be able to induce differentiation of ESCs into insulin-producing cells and pre-differentiation of mS-MSC. Support: FAPESP, CNPq, FINEP, MS-DECIT, MCT, BNDES. Key words: beta-cell, Diabetes, murine embryonic stem cells, endoderm inducers, murine skin-derived mesenchymal stem cells.

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Poster Board Number: T-1137

PRODUCTION OF INSULIN-PRODUCING CELLS DERIVED FROM MOUSE ES CELLS THROUGH SERUM-FREE-INDUCED DEFINITIVE ENDODERM

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One promising approach for the development of cell therapies for diabetes treatment is to utilize pancreatic endocrine cells differentiated from pluripotent stem cells such as embryonic stem (ES) cells. Here, we show an original and novel method to induce the differentiation into insulin-producing cells from mouse ES (mES) cells. For the efficient production of mature insulin-producing cells, we hypothesized that induction of definitive endoderm (DE) is the first important step. We have focused on serum free induction as an effective method to direct mES cells to DE. First, a serum free medium was utilized to induce (DE) formation. The formation of DE was strongly supported by the quantitative RT-PCR analysis showing the expression of endoderm markers Sox17 and Foxa2 and a DE specific marker Cxcr4 increased over the course of DE induction. The differentiated endoderm cells were then treated with KGF and all-trans retinoic acid (RA) to induce pancreatic specialization which was confirmed by the expression of the early pancreatic transcription factor Pdx1. Finally, the differentiated cells were transferred onto low-adhesion dishes for suspension culture in maturation medium containing nicotinamide and Ex-4. The differentiated cells in this stage were formed spherical clusters and expressed Insulin as determined by RT-PCR. Furthermore, the result of immunostaining analysis proved that the differentiated cells expressed Insulin, C-peptide, and Pdx1. The ability of glucose-stimulated insulin secretion (GSIS) of the differentiated cells at this stage was measured by ELISA. The differentiated cells indeed secreted insulin in response to glucose stimuli. These results suggested that our approach can be a novel and effective method to produce mature insulin-producing cells, paving the way for the development of cell therapies utilizing pluripotent stem cells for diabetes treatment.

Poster Board Number: T-1138

MICRORNA SIGNATURES AND FUNCTIONS OF *IN VITRO* GENERATED PANCREATIC BETA ISLET CELLS

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Insulin-expressing beta islet-like cells differentiated from human embryonic stem cells (hESCs) are a model system for studying the molecular signaling pathways underlying beta cell development. MicroRNA repression of mRNAs is an important mechanism that regulates gene expression. We performed genome-wide microarray profiling of microRNA and mRNA expression at 6 cell stages during *in vitro* directed differentiation of hESCs into beta islet-like cells _ undifferentiated hESCs, definitive endoderm, primitive gut tube, posterior foregut, pancreatic progenitor and hormone-expressing endocrine cells, in addition to human fetal pancreatic tissue samples. Our results showed that both microRNAs and mRNAs are expressed in a dynamic pattern, and that distinct groups of microRNAs were co-expressed during differentiation. Using supervised group-wise statistical analysis and semi-supervised hierarchical clustering, we found that these microRNAs fell into several groups. There was a large cluster of pluripotency-associated microRNAs located on chromosome 19q13 (mir-448, -498, -512, -518, -519, -520), that was rapidly down-regulated upon induction of differentiation. Another pluripotency-associated cluster on chromosome 4q25 (the mir-302 family) displayed persistent expression until the later stages of differentiation. Similarly, the microRNAs that showed increased expression with differentiation also followed different time courses of induction, with some (mir-375, -26b,-200c) induced quickly upon differentiation and remaining high, while others (mir-30d, -24,-27b) were induced only at later stages. To understand the functions of microRNAs during differentiation, we performed integrated analysis of the microRNA and mRNA profiling data generated from the same samples using a web-based bioinformatic tool followed by permutation-based statistical testing for significance. Our results showed that undifferentiated hESC-enriched microRNAs were predominantly negatively correlated with their mRNA targets, while differentiation-enriched miRNAs and their targets tended to be positively correlated. These microRNA/mRNA pairs were further investigated using luciferase reporter assays, which identified dual roles for the miR-200a/141 family of microRNAs in regulating both the mesenchymal-to-epithelial transition and definitive endoderm formation during early differentiation; at later stages of differentiation miR-30d and let-7e regulated the pancreatic progenitor gene RFX6. Samples from the later stages of differentiation clustered closely with fetal pancreas samples, but a comparison across all samples showed differences between tissues and cells that may reflect a less differentiated or less mature state in hESC derived cells. In summary, this study has used *in vitro* directed differentiation of hESCs as a model system to identify specific microRNAs and mRNA targets that are involved in regulation of beta cell differentiation.

Poster Board Number: T-1139

VISUALIZATION OF PANCREATIC CANCER STEM CELLS TO DEVELOP NOVEL TARGETING THERAPY

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BACKGROUND Increasing evidence suggests that cancers contain a small subpopulation of cells having the ability to self-renew and differentiate into multiple phenotypes, also known as cancer stem cells (CSCs). One of CSCs characters is its resistance to chemo and/or radiation therapy. Identification and purification of cancer stem cells (CSCs) by cancer stem cell markers should offer a clue of the therapeutic targets, but the result is still unstatisfactory. Here in this study, we successfully applied monitoring system based on the CSC-spesific function, and performing drug screening to discover compounds that selectively targeting pancreatic CSCs. METHODS Like normal stem cells, CSCs are usually quiescent with low protein turnover, reduced metabolism and downregulation of proteasome activity. Based on these characteristics, we used the monitoring system of human pancreatic cancer stem cells which express high green fluorescent. Flow cytometry and cell sorting was useful to isolate cells with high green expressing cells (CSChigh) from low green expressing cells (CSClow). To evaluate the stem cell characteristics, observation by time-lapse microscopy, sphere formation assay, drug sensitivity assay, and *in vivo* tumorigenicity assay were performed. Drug screening of both cells was performed to discover compounds that selectively targeting pancreatic CSCs. RESULTS: In our monitoring system, the high green expressing cells (CSChigh) population existed in approximately 0.5 % of the human pancreatic cancer cell.. Asymmetric division of CSCigh cells into CSClow and CSChigh cells were clearly recognized, while CSClow cells did never divide into Gdeghigh cells. CSChigh cells could form complete

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spheres while the CSClow cells could not form such spheres. In in vivo tumorigenicity assay, CSChigh formed tumors with only 10 cells indicating the remarkable tumorigenecity of CSChigh cells. CSChigh cells were resistant to gemcitabine treatment, while CSClow cells were highly sensitive to this treatment. We performed a drug screen on both cell types and successfully identify a novel compound targeting specifically the pancreatic CSCs. Anti-CSC effects of the compound were confirmed by in vitro dynamic images and in vivo tumor analyses. CONCLUSION: In our system, the high green expressing cells (CSChigh) were proved to have cancer stem-like characteristics. The visualization system can be applicable to direct monitoring of drug sensitivity in the specific CSCs, distinct from non-CSCs. Our studies indicated rational application of drug screening to discover compounds targeting selectively the pancreatic CSCs. Further studies using this system may improve therapeutic approaches for aggressive cancer and subsequently outcomes of the patients in the future.

Poster Board Number: T-1140

MODELING OF PERMANENT NEONATAL DIABETES MELLITUS USING PATIENT-SPECIFIC IPS CELLS

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Permanent neonatal diabetes mellitus (PNDM) has been associated with monogenic defects in about half of the studied cases. Among 12 PNDM cases studied in the genetically isolated Finnish population, mutations in the best-known genes (ABCC8, KCNJ11, Insulin) have been found in only 3 of them, suggesting the prevalence of other mutations not yet identified. The generation of in vitro models using patient-derived induced pluripotent stem cells (iPSC) constitutes a valuable tool for the study of disease mechanisms. We aim to use this approach to establish a platform enabling studies of pathogenetic mechanisms in PNDM. Fibroblasts derived from PNDM patients and healthy donors were efficiently reprogrammed into iPSCs using retroviral or Sendai virus delivery of reprogramming factors Oct4, Klf4, Sox2 and c-Myc, in combination with sodium butyrate. Generated iPSC lines showed the characteristic morphology and markers of pluripotent stem cells and they were able to differentiate into the three embryonic germ layers in teratoma assay. Derivation of substantial numbers of fetal stage pancreatic islet cells from human pluripotent stem cells is possible by mimicking in vitro the developmental pathways occurring *in vivo*. We have optimized a feeder- and serum-free, multistage pancreas differentiation protocol with the incorporation of TGFβ/BMP signaling pathway inhibitors at different stages. Healthy control iPSCs were successfully differentiated towards pancreatic endocrine lineage. Quantitative PCR (qPCR) analysis revealed proper differentiation kinetics, showing the upregulation of FoxA2, Sox17 and CXCR4 expression during the definitive endoderm induction stage, and Pdx1, Nkx6.1 and Ngn3 in the endocrine progenitors stage. Cytometric analysis showed efficient specification of definitive endoderm stage cells, with 75%-90% of CXCR4+ cells. Colocalization of Pdx1 and Nkx6.1 was used to verify the islet progenitors, which were present in the mid stages of our differentiation protocol. In the later stages, immunocytochemistry revealed abundant clusters of cells single or double positive for insulin/c-peptide and glucagon. These results were confirmed by qPCR, demonstrating the generation of fetal-stage pancreatic islet cells using this protocol. These results demonstrate that it is feasible to establish a disease modeling platform based on the pancreatic differentiation of iPSC derived from PNDM patients. The generation and analysis of iPSC lines with mutations in well-studied genes (KCNJ11, Insulin) is currently ongoing to validate this experimental approach. In the next stage, the analysis will be performed in cases with unknown etiology. In combination with next generation sequencing, this is expected to enable the identification of new causes for human diabetes.

Poster Board Number: T-1141

CD133 IS INVOLVED IN MIGRATION AND INVASION THROUGH REGULATION ON N-CADHERIN IN PANCREATIC CANCER

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Pancreatic cancer is the fifth leading cause of cancer-related death in Japan and the fourth in the United States. Over 90% of pancreatic cancer-associated mortality is due to metastasis, which involves several serial steps from disseminating from primary cancer to colonizing in the distant organ. CD133, a marker of cancer stem cells (CSCs) in various solid tumors including pancreatic cancer, has been studied for recent decade. However, the role of CD133 is still obscure. Therefore, we investigated the CD133 functions using human pancreatic cancer cell line, Capan-1. In our experiments, the tumorigenesis of CD133-positive pancreatic cancer cells was higher than that of CD133-negative cells using NOD/SCID mice. On the other hand, it has been supposed that the epithelial-mesenchymal transition (EMT) is one of CSC properties in the process of metastasis. We recently established the high migratory subclone, Capan1M9, from Capan-1 cells. Capan1M9 cells showed a three-fold increase in migration and invasion compared to the parental cell line (Capan-1). Furthermore, the higher expressions of Slug, N-cadherin and fibronectin which are components of characteristic of EMT were shown in this Capan1M9 cell line. Importantly, CD133 expression also elevated in the Capan1M9 cells by the flow cytometric analysis and western blot examinations. Subsequently, shRNACD133 was transfected into Capan1M9 to knockdown CD133 expression. This result showed the reduction of migratory and invasive abilities of the Capan1M9 cells and the repression of Slug and N-cadherin expression. In contrast, there was no difference not only on spheroid formation but also xenograft tumor growth between Capan1M9 and shRNACD133Capan1M9. In addition, N-cadherin was down regulated but CD133 expression showed no influence after shRNAslug transfection into Capan1M9. Next,ERK inhibitor, U0126, was administrated into Capan1M9 to determine whether ERK pathway involved in N-cadherin regulation. We found that not only N-cadherin also Slug and CD133 were down regulated significantly by U0126 administration. Phosphorylated ERK1/2 could be blotted among N-cadherin immunoprecipitated elute. Taken together, ERK, CD133 and Slug interact as a modulation loop on N-cadherin expression. Further study should shed new light to understand the orchestrated network associated with CD133 of pancreatic cancer metastasis. These insights on CD133 regulation could be promising the novel targeted therapy for pancreatic cancer.

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Poster Board Number: T-1142

FIGF PLAYS AN IMPORTANT ROLE IN THE PANCREAS REGENERATION

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Objective To explore the role of Figf (C-fos induced growth factor) in pancreas regeneration. Methods Mouse model of pancreatic regeneration was established by pancreatectomy. Pancreatic function loss and regeneration was monitored by time-coursed blood glucose testing after pancreatectomy. Regenerating pancreas tissues were collected 48h post pancreatectomy for RNA isolation. Gene expression profiling was performed using mouse whole genome gene chips. Differentially expressed genes were verified by qPCR. The target gene, Figf, was selected and cloned in expression plasmid. MS1 cells cultured *in vitro* were transfected with the Figf constructs, and insulin secretion by the transfected cells was detected by ELISA 36h after cell transfection. The mRNA levels of Pdx1 and Insulin1 genes in transfected cells were tested by qPCR. Results Figf expression was significantly increased in regenerating pancreas. Comparing with untransfected and vector-transfected groups, insulin secretion in Figf transfected cells increased significantly (116.89±6.09 pg/ml over untransfected group, P<0.01;and 114.24±4.60 pg/ml over vector- transfected group,P<0.01). Again, comparing with untransfected and vector-transfected groups, Figf over expression group expressed significantly higher mRNA levels of Pdx1 and Insulin1 genes(P<0.01). Conclusion: Over expression of Figf gene in MS1 cells can elevate the expression of Pdx1 and Insulin1 in mRNA levels, and increase insulin secretion. Figf may be one of the key genes evolved in pancreas regeneration and play an important role in β-cell transition regeneration. Correspondence: Li yukui E-mail: yukuili@hotmail.com(The research is supported by the National Natural Science Foundation of China, NO. 81160098)

Intestinal/Gut Cells

Poster Board Number: T-1143

FUNCTIONAL ANALYSIS OF SNAI1 IN THE MOUSE INTESTINAL STEM CELL NICHE

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The Snail family of transcriptional repressors have a key role in mediating epithelial to mesenchymal transitions during both embryonic development and cancer metastasis. Although they are generally regarded as markers of mesenchymal cells, Snail proteins have also recently been implicated in regulating stem cell populations in several organs. We have examined the role of Snail proteins in the mouse intestinal epithelium that is continuously renewed via a population of multipotent stem cells that reside in the base of crypts. We have investigated Snai 1 function in the intestinal stem cell niche using an inducible conditional knockout and found that Snai 1 is required for maintenance of the crypt base columnar stem cell population. We have also analysed the effects on the crypt base columnar stem cell population using a combination of Fluorescent Activated Cell Sorting (FACS) and organoid culture. Further analysis of the tissue of Snai1 mouse models has revealed an effect on the differentiation of mature cell lineages. In conclusion, these results suggest that Snai1 has a key role in stem cell maintenance and control of cellular differentiation.

Poster Board Number: T-1144

COMMITTED BUT NOT BEYOND RECALL: CONDITIONAL CLONOGENICITY OF THE MURINE INTESTINAL LABEL RETAINING CELL

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The intestinal epithelium is maintained by rapidly cycling stem cells located at the base of the crypts of Lieberkuhn and characterised by the expression of Lgr5. These cells generate clones that follow characteristic neutral drift behaviour demonstrating that during homeostasis these cycling Lgr5 expressing cells represent an equipotent pool of progenitors. For several decades label retaining quiescent cells have been shown to exist in the base of crypts but their nature and function have been difficult to elucidate. Recently, several reports utilising reverse genetics have overlaid stem cell marker expression with label retaining cells and in parallel shown stem cell capacity from the same marked cells. However none of these markers localise solely to label retaining cells. In contrast, we decided to isolate and characterise quiescent cells based on their inherent property of label retention. Ah H2B-eYFP mice were generated to allow ex-vivo functional and molecular characterisation of label retaining cells via pulse chase and fluorescence activated cell sorting. Further, using a combination of *in vitro* techniques and a novel dimerisable Cre recombinase transgenic mouse we have definitively demonstrated the nature of intestinal label retaining cells in both normal homeostasis and times of epithelial injury. Our results show the intestinal label retaining cell to represent a secretory cell precursor committed to the Paneth and enteroendcorine cell lineages but which is capable of recall to the stem cell compartment during times of epithelial damage. Using similar techniques we have also shown the existence of quiescent cells in mouse intestinal tumours. These cells share many characteristics to those of normal intestinal label retaining cells in relation to expression profile and clonogenic potential. These data have significant implications for the modern management of colorectal cancer.

Poster Board Number: T-1145

GENERATION OF INTESTINAL TISSUE FROM INDUCED PLURIPOTENT STEM CELLS

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Introduction: Crohn's Disease (CD) is a chronic inflammatory disorder that can affect any part of the gastrointestinal tract. Despite extensive research, the causes of CD remain elusive. One relatively unexplored area of study is what role the intestinal epithelium may play in this disease. The intestinal epithelium, which contains four differentiated cell types, has been poorly studied as it had previously not been possible to study it *in vitro*. Recently it was found that induced pluripotent stem cells (iPSCs) could be directed to form intestinal tissue, which contained all the four cell types, *in vitro* (Spence et al, 2011). Method: The generation of intestinal tissue from iPSCs is a multi-step process which involves directing iPSCs into definitive endoderm, then hindgut tissue and then subsequently into three dimensional intestinal "organoids." To generate definitive endoderm, two control integration-free iPSC lines were incubated with 100ng/ml of Activin A under low serum conditions

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for three days. To generate hindgut tissue, definitive endoderm was incubated in Advanced DMEM/F12 media with 10% FCS with or without 500ng/ml of Wnt3A and FGF4 for a further three to four days. During the hindgut differentiation protocol, epithelial "tubes" became visible after three to four days. These epithelial tubes were harvested and then cultured in a three dimensional Matrigel matrix containing noggin, RSpondin-1, EGF and B27. Results: The generation of definitive endodermal tissue was confirmed by the upregulation of Sox17, FoxA2 and Goosecoid mRNA and by immunocytochemistry which showed the co-expression of Sox17 and FoxA2 in the vast majority of these cells. The generation of hindgut tissue was demonstrated by both the presence and upregulation of the hindgut intestinal marker CDX2. The epithelial tubes that were cultured in a three dimensional Matrigel matrix steadily increased in size over time to generate spherical intestinal organoids (approx. 500µm). After 14 days, the organoids contained CDX2+ cells which also expressed the early proliferating intestinal cell markers, KLF5 and Sox9. The four differentiated intestinal cell subtypes were also found in these organoids. Goblet cells (Muc2+), Paneth cells (Lysozyme+), enterocytes (FABP2+) and enteroendocrine cells (Chromogranin A+) were all observed in these intestinal organoids at various different timepoints. Conclusion: Intestinal tissue containing the four differentiated cell types can be generated from control iPSCs. iPSCs will now be generated from CD patients with genetic variations in intestinal cell proteins. These iPSCs will be then be directed to form intestinal organoids. Such organoids may reveal the consequence of these mutations in the intestinal epithelium and may ultimately elucidate some of the mechanisms involved in the pathogenesis of CD.

Poster Board Number: T-1146

LGR5 INTESTINAL STEM CELLS AND THEIR MAINTENANCE

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The leucine-rich repeat containing G protein-coupled receptor 5 (Lgr5) marks actively cycling crypt base columnar cells (CBCs) that are multipotent, long-lived, and are largely responsible for homeostatic regeneration in the intestinal epithelium. Recently, the G-protein-coupled receptors Lgr4-6 have been reported to associate with Wnt receptors to mediate R-spondin signaling. Although Paneth cells have been suggested to serve as niche cells for Lgr5+ intestinal stem cells (ISCs), perhaps through secretion of essential paracrine factors, the precise mechanisms regulating maintenance of Lgr5+ ISC remain poorly understood. We have used adenovirus to overexpress the soluble ligand-binding Lgr5 ectodomain in the circulation of adult mice. Circulating Lgr5 ectodomain induced the migration of Paneth cells from the crypts, their eventual loss, and a concomitant disappearance of Lgr5+ ISCs. Paneth cell migration was associated with downregulation of Wnt signaling and its target EphB3. The loss of Lgr5+ ISCs did not affect maintenance of the intestinal epithelium, nor did Lgr5+ stem cells disappear from non-intestinal organs. The use of this adenoviral system to examine the relationship of Lgr5+ ISC to other intestinal stem cell populations will be discussed. Together, these findings characterize an easily tractable experimental model for the *in vivo* deletion of Lgr5+ ISC, and suggest that Lgr receptors function to actively maintain Lgr5+ ISCs *in vivo*.

Poster Board Number: T-1147

COMPUTATIONAL ANALYSIS OF STEM AND NICHE CELL PATTERNS INSIDE INTESTINAL CRYPTS

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INTRODUCTION The intestinal epithelium is the most rapidly dividing tissue in the body, turning over every 3-4 days. Each intestinal crypt contains a few slow-cycling BMI1+ intestinal stem cells (ISCs) at the +4 position and 12-14 faster-cycling Lgr5+ ISCs. At the base of the crypt, the Lgr5+ ISCs and CD24+ Paneth cells form checkerboard patterns, which provide the niche for ISC self-renewal. Subversion of this mechanism leads to dysplasia and cancer Because Paneth cells express the Notch ligand DLL4 while ISCs express the Notch1 and 2 receptors, it has been postulated that Notch-dependent lateral inhibition (NDLI) - circuits in which Notch signaling in a cell downregulates signaling in its neighbors - is responsible for the checkerboard pattern between ISCs and Paneth cells. However, the exact mechanism remains unclear. Here we used analytical tools from systems biology to quantitatively investigate alternative NDLI circuits that can impact ISC-Paneth pattern formation and maintenance. RESULTS We first examined a transcriptional feedback as the potential NDLI mechanism in the crypt. In this transcriptional feedback, Notch ligands on one cell activates Notch receptors on an adjacent cell, which leads to downregulation of Notch ligands DLL1 and DLL4 through the transcriptional factor MATH1. Steady sate analysis of an ordinary differential equation (ODE) based model showed that this feedback is sufficient to generate checkerboard-like patterns. However, sensitivity analysis revealed that the resulting pattern is not robust to variations and perturbations. Ensuing dynamical analysis of the pattern further revealed that the pattern is not sufficiently stable in terms of Maximal Lyapunov Exponent (MLE) and that it is a slow process to form a steady pattern. In contrast, the base of the crypt is a dynamic environment, wherein Lgr5+ ISCs constantly divide and leave the niche to replace the entire crypt epithelium every 3~5 days. Therefore, the transcriptional feedback is not likely the only NDLI mechanism responsible for regulating the checkerboard pattern. We then examined a post-translational feedback as an alternative or secondary NDLI mechanism to the transcriptional feedback. In this post-translational feedback, Notch receptors and ligands interact in the same cell, and the receptor-ligand complexes are mutually inhibited and degraded by endocytosis (cis-inhibition). Time-lapse movies of single cells with fluorescent reporters showed that cis-inhibition acts as an ultra-sensitive switch that amplifies the small difference in ligand levels between adjacent cells. Same steady-state and dynamic analyses demonstrate that the post-translational feedback through cis-inhibition is a more robust mechanism for generating and maintaining the checkerboard pattern between ISC and Paneth cells. Compared to the transcriptional feedback, cis-inhibition leads to speedier pattern formation and more stable patterns. CONCLUSION Our computational analyses suggest that the MATH1-mediated transcriptional feedback is insufficient to generate robust checkerboard patterns, so the post-translational feedback through cis-inhibition is likely an active NDLI mechanism inside the crypt. METHOD Sensitivity analyses were performed using the Matlab SimBiology toolbox. Stability and speed of pattern formation was analyzed using MLE and stochastic simulations. The speed of pattern formation was analyzed using stochastic simulations with different initial conditions.

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Poster Board Number: T-1148

DOSE-DEPENDENT ROLES FOR CANONICAL WNT SIGNALING IN *DE NOVO* CRYPT FORMATION AND CELL CYCLE PROPERTIES OF COLONIC EPITHELIUM

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Canonical Wnt signaling plays a pivotal role in the physiology and carcinogenesis of the intestinal epithelium. Notably, the strength of Wnt signaling has been reported to affect the tumor spectrum in mice, and it controls hematopoietic stem cell self renewal vs. differentiation. Whether intestinal stem cells also respond to Wnt signaling in a dose-dependent manner remains unknown. Here, we investigated the effect of canonical Wnt activation on colonic epithelial differentiation by controlling the expression levels of stabilized β-catenin using a doxycycline-inducible transgenic system in mice. We show that elevated levels of Wnt signaling induce the amplification of Lgr5+ crypt stem cells, which is accompanied by crypt fission and a reduction in cell proliferation among progenitor cells. In contrast, lower levels of β-catenin induction enhanced cell proliferation rates of epithelial progenitors without affecting crypt fission rates. Notably, slow-cycling cells produced by β-catenin activation exhibit activation of Notch signaling. Consistent with the interpretation that the combination of Notch and Wnt signaling maintains cells in a low proliferative state, the treatment of β-catenin expressing mice with a Notch inhibitor turned such slow-cycling cells into actively proliferating cells. Our results indicate that the activation of the canonical Wnt signaling pathway is sufficient to amplify colonic stem cells, and suggest that different levels of canonical Wnt activations, in cooperation with Notch signaling, establish a hierarchy of slower-cycling stem cells and faster-cycling progenitor cells characteristic for the colonic epithelium.

Muscle Cells

Poster Board Number: T-1151

HTS ASSAY WITH MESODERMAL PROGENY CELLS DERIVED FORM HUMAN PLURIPOTENT STEM CELLS (HES AND IPS) FOR THE IDENTIFICATION OF MEVALONATE PATHWAY MODULATORS

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The mevalonate pathway provides cells with essential products such as cholesterol and substrates for isoprenylation and is therefore is critical for cell growth and differentiation. Designed HTS cell assays for the identification of modulators mevalonate pathways are important to identify new functional inhibitors, to increase our knowledge on the mevalonate pathway and to predict and prevent toxic effects. One of the key factors for success of HTS bioassays is the source of the cells used for the screen. In this regard, human pluripotent stem cells possess numerous advantages: a human origin, an unlimited growth potential, a genetic stability and the capacity to differentiate into all the cell types forming an organism. For this study, we defined a simple and robust differentiation protocol to obtain large and homogeneous amounts of mesodermal progenitor cells (MPCs) from human pluripotent stem cells (hES and iPS Herein, we describe the optimisation of MPC culture conditions in multiwell dishes for the implementation of a cell-based assay for primary screening by HTS in presence or absence of mevalonate to screen functional inhibitors of HMG-CoA reductase. The screening of the Prestwick library, consisting of 1120 highly diverse FDA-approved drugs that have established biological activities demonstrates specificity and robustness of our HTS assay. In each screen, we were able to select the three HMG-CoA reductase inhibitors (lovastatin, fluvostatin, simvastatin) present in the Prestwick library. EC 50 determination of statins confirmed the toxicity of this class of molecules on MPCs and revealed that MPCs display a toxicity similar to the one observed with adult muscle cells, a major target of statins sideeffect. Furthermore, we have designed a second type of cell bioassay to identify molecules,that protect cells from statin induced toxicity induced . In this cell bioassay, we identified by screening more than 10 000 molecules, two classes of molecules that protect from simvastatin toxicity. Mechanistic explorations indicate that one of these molecules control the level of HMG-CoA reductase transcript. MPCs derived from hES and iPS were equally potent as a source of cells to identify hits by HTS in both bioassays. In conclusion, differentiated mesodermal progenies derived from iPS and hES allow the identification by HTS of functional inhibitors of HMG-CoA reductase and molecules which can rescue from simvastatin toxicity by controlling the expression of mRNA coding for HMG-CoA reductase.

Poster Board Number: T-1152

AGE-DEPENDENT EFFECTS OF PRO-INFLAMMATORY CYTOKINES ON HUMAN MYOBLAST DIFFERENTIATION

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Objective: Targeted enhancement of proliferation and differentiation of muscle progenitor cells is a key strategy for combating age-related muscle wasting, weakness and sarcopenia. As the pro-inflammatory circulating cytokines are elevated in many age-related disorders, we have investigated the action of TNF-α, IL-1β and IL-6 on proliferation and differentiation of muscle progenitor cells (myoblasts) of individuals with different ages: young-less than 30 and elder -over 65 years. Methods: Primary cultured human skeletal muscle myoblasts were purified with CD56 antibodies microbeads on MACS (Miltenyi Biotec) and cultured in the presence of HGF. Serum free cultures we stimulated for 6-7 days with each cytokine (PeproTech) alone or in different combinations with differentiation media supplement: insulin- transferrin-sodium selenite /ITS/(Sigma-Aldrich). Differentiation was estimated by monitoring myotube formation, fusion index and % of myonuclei. Results: The myoblasts cultivated from older individuals differentiated markedly slower than myoblasts from young individuals in ITS medium. Treatment of human myoblasts with TNF-α and IL-1β increased the proliferation and block the differentiation even in the presence

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of differentiation media supplement (ITS). IL-6 alone induced the differentiation at the same level as ITS and there was no remarkable co-effect of IL-6 with ITS detected. The mixture of cytokines (TNF-α, IL-1β and IL-6) with ITS induced mainly the proliferation with remarkable decrease of differentiation as compared to effects of ITS or IL6 alone. Conclusion: There exist age related differences in cytokine IL-6 action on myoblasts derived from young and old individuals. Compared to myoblasts from young individuals the differentiation was suppressed in ITS- treated cultures as well as in cultures with ITS+IL6 and IL6 alone in old individuals. Variation of myoblast differentiation in elder individuals was much more remarkable than in young individuals. IL-1β and TNF-α suppressed the differentiation of myoblasts and the action was quite similar in both investigated group.

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NOVEL HUMAN ARTIFICIAL CHROMOSOMES AND TECHNOLOGIES FOR STEM CELL-BASED THERAPIES OF DUCHENNE MUSCULAR DYSTROPHY

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We recently showed that stem cell-mediated human artificial chromosome (HAC) transfer ameliorates the pathology in a mouse model (mdx) of Duchenne muscular dystrophy (DMD), which is caused by mutations in the large dystrophin gene (2.4 Mb). HACs are stably maintained in the host cell as episomal vectors, like native chromosomes. Their capacity to carry large genomic loci containing regulatory elements allows physiological regulation of the introduced genes. We succeeded in developing a HAC vector containing the entire human dystrophin gene (DYS-HAC1), which was then successfully transferred into mesenchymal, embryonic and induced pluripotent stem cells). Importantly, DYS-HAC1 was also transferred into mesoangioblasts (vessel-associated stem cells) derived from the dystrophic mdx mouse. Upon intramuscular or intra-arterial transplantation of dystrophin-corrected mdx mesoangioblasts into mdx mice, donor cells gave rise to large clusters of dystrophin-positive myofibers together with donor-derived satellite cells, which significantly ameliorated morphology and function of dystrophic muscles. However, in order to translate this approach to DMD patients, there are still many technical hurdles that need to be overcome. Here we report novel strategies and technologies of HAC-mediated cell therapy for DMD patients including: 1) Transfer of novel DYS-HACs into human mesoangioblasts; 2) HAC-mediated enhancement of skeletal muscle differentiation and 3) higher dystrophin expression. 1) In order to extend the proliferative capability of DMD mesoangioblasts to survive selection after HAC transfer; we developed a platform for their engineering by means of excisable lentiviral vectors expressing immortalizing genes (hTERT and Bmi1) in a reversible fashion. Alternatively, we have also derived mesoangioblasts from iPS cells. Both approaches generate cells able to be genetically corrected with novel DYS-HACs with reduced immunogeneicity (no EGFP, TK, Blasticidine and hprt at variance with DYSHAC1). 2) For effective skeletal muscle conversion of target cells, a DYS-HAC containing an inducible MyoD gene (MyoD-ER) was developed (DYS-HAC3). 3) Dystrophin gene dosage of transplanted-cells containing single DYS-HAC is not close to wild-type dystrophin because of fusion with endogenous myofibers (containing hundreds of nuclei) that do not express dystrophin. To increase dystrophin expression level, we transferred three novel DYS-HACs into mdx mesoangioblasts, each one containing a different conditional selection marker gene (DYS-HAC2: neomycin; DYS-HAC4: blasticidin; DYS-HAC6: histidinol dehydrogenase) developed using homologous recombination. Mdx mesoangioblasts containing multiple DYS-HACs (up to 3) were developed and their dystrophin expression is being tested. Thus, these DMD-specific DYS-HACs and technologies are expected to speed up future translational gene and cell therapies for muscular dystrophy.

Poster Board Number: T-1154

ADAR1 REGULATES THE MYOGENIC PROGRAM IN MOUSE SKELETAL MYOBLASTS

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ADAR1 (adenosine deaminase acting on RNA 1) catalyzes the deamination of adenosine to inosine on RNA molecules of double stranded structure. Two isoforms of ADAR1 are known, both of which possess RNA-editing activity: an interferon (IFN) inducible 150 kDa protein (p150) and a constitutively expressed amino-terminally truncated 110 kDa protein (p110). The p150 isoform is found in both the cytoplasm and nucleus, while the p110 protein is localized predominantly in the nucleus. A-to-I RNA editing not only affects targeted transcripts by altering the sequence and/or structure of the encoded products, but also serves to regulate retrotransposons and gene silencing. In this study, we identified a novel aspect of biological function of ADAR1 in the regulation of skeletal myogenesis. We have investigated ADAR1 expression during myoblasts-myotubes differentiation. ADAR1 p150 expression transiently increased during cell confluence and initial differentiation process, whereas the expression of both ADAR1 forms dropped in the differentiated myotubes. Furthermore, we discovered that ADRA1 p150 transient up-regulation in early differentiation is mediated by the IFN signaling pathway-associated interferon stimulated response element (ISRE) and subsequently repressed by the myogenic transcription factor MyoD. Intriguingly, knockdown of ADAR1 or overexpression of a catalytic mutant reduced the expression of myogenesis-associated markers, demonstrating the significance of ADAR1-mediated nuclear RNA editing in the early myogenic program. On the other hand, the function of ADAR1 p150 in early stage of differentiation possibly lies in the regulation of PKR-mediated apoptosis. Next, we demonstrated that in the late stage of myogenesis, miR-1/206-mediated inhibition facilitated the down-regulation of ADAR1 proteins. However, ectopic ADAR1 over-expression at this stage caused abnormal myotube formation, further implying stage-specific functions of ADAR1 in the myogenic process. Taken together, our results pointed to the scenario that ARAR1-mediated regulation, possibly through editing of as yet unknown transcripts, is essential for scheduled progression of skeletal myogenesis.

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THE INFLUENCE OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR Γ1 ON DIFFERENTIATION OF MOUSE EMBRYONIC STEM CELLS TO CARDIAC BEATING CELLS

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Peroxisome proliferator activated receptor γ, a member of nuclear ligand activated transcription factors PPARs, exerts various metabolic functions and also affects differentiation process. To testify the importance of PPARγ in cardiac differentiation of mouse embryonic stem cells (mESCs), in the first step, its expression level was assessed during cardiac differentiation of mouse embryonic stem cells. Data revealed an elevation in expression level of PPARγ when beating bodies (BBs) were formed. Subsequently, involvement of PPARγ in during and post-cardiac precursor cells (CPs) formation was examined by application of agonist (Rosiglitazone, 5μM) and antagonist (GW9662, 10μM). Our results indicated that PPARγ inactivation via treatment with GW9662 during CPs formation, reduced expression of cardiac beating bodies' markers. However, PPARγ inactivation by antagonist treatment post-CPs formation stage did not affect beating bodies' differentiation. Here, we have demonstrated a stage dependent role of PPARγ modulation on cardiac differentiation of mESCs for the first time.

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TRANSPLANTATION OF MESENCHYMAL STEM CELLS DERIVED FROM ES CELLS PROMOTES MUSCLE REGENERATION; RE-INNERVATION AND THEREBY ACCELERATES FUNCTIONAL RECOVERY OF INJURED SKELETAL MUSCLE

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Background and Aims: It is well known that mesenchymal stem cells (MSCs) have a high potential for differentiation into mesenchymal cells. Adipose tissues have been proved as a useful and rich source of adipose tissue-derived mesenchymal stem cells (ADSCs). Although ADSCs have shown therapeutic efficiency in repairing damaged mesenchymal tissues, their isolation and purification from adult adipose tissue still involves complicated and troublesome procedures. Furthermore, they readily differentiate into adipocytes, osteocytes and chondrocytes but not into muscular cells. Mouse ES cells, on the other hand, are pluripotent, and their induction to adipogenesis has been well described. Then, we currently established mesenchymal stem cells originating from mouse ES cells (E-MSCs) that showed markedly higher potential for differentiation into skeletal muscles in vitro than common MSCs and to promote functional recovery of injured skeletal muscle, and presented at 9th ISSCR (2011 at Toronto). Here we evaluated the potential of E-MSCs for differentiation into skeletal muscles in vivo, morphological regeneration, and functional recovery of injured muscles by the transplantation of E-MSCs. Their transplantation promoted the functional recovery of injured muscles by an acceleration of muscle regeneration and a re-innervation of peripheral nerves. Materials and Methods: ES cells (G4-2; kind gifts from Dr. Niwa) carrying the enhanced green fluorescent protein _EGFP_ gene under the control of cytomegalovirus/chicken β-actin promoter were expanded their population and embryoid bodies (EBs) were formed in hanging drops. EBs were cultured in a retinoic acid containing medium. After washing, they were settled on culture dishes and maintained with adipogenesis medium (insulin / triiodo-thyronine). After the increase of CD105+ cells, we isolated and sorted them by a magnetic cell sorter (MACS; Miltenyi). CD105 positive E-MSCs were transplanted into the injured tibialis anterior muscles of SCID mice 24 h after clamping. After 1, 2, 3, and 4weeks of transplantation, the myogenic differentiation of M-ESCs, muscle regeneration, and re-innervation were analyzed. The myogenic differentiation was examined by Pax7, M-cadherin and/or skeletal muscle myosin heavy-chain (MHC) immuno-staining, and re- innervation was examined by α-Bungarotoxin and SMI-31, respectively. We, then counted EGFP expressing E-MSCs among regenerating muscle cells and innervation of peripheral nerves. Furthermore functional performance of transplanted animal and sham transplanted animals were compared using a functional analyzer Cat Walk XT (Noldus). Results and Conclusion: When E-MSCs were transplanted into the injured tibialis anterior muscles of SCID mice, most of them differentiated into skeletal muscles *in vivo*. The transplantation of E-MSCs promoted a functional recovery of injured muscles by an acceleration of muscle regeneration and a re-innervation of the peripheral nerves. Thus, cell therapy using MSCs derived from pluripotent stem cells is one of the most effective and safe ways to improve functional recovery of damaged skeletal muscles.

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AUTOPHAGY IS ESSENTIAL FOR MYOFIBRIL DIFFERENTIATION.

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Introduction. Cardiovascular diseases constitute the leading cause of mortality and morbidity worldwide, with myocardial infarction responsible for more than 10% of deaths. Although patients affected by heart failure can be treated with transplantation, the ever increasing patient need has not been met with a corresponding increase in donor organs. Over the last decade, stem/progenitor cell therapy has emerged as an innovative approach to overcome this limitation with the potential to provide cardiac repair and regeneration. Efforts to regenerate myocardium through differentiation of cardiac stem cells to fully differentiated cardiomyocytes have met with limited success. Autophagy is a lysosome-mediated degradation pathway used by eukaryotes to recycle cytosolic components in both basal and stress conditions. Recently, autophagy has been recognized to be important for metabolic reprogramming during cell differentiation. Tissue specific gene-targeting studies have revealed that autophagy functions in several specific lineages, including adipocytes, erythrocytes, T cells, and B-1a cells. Autophagy may play an important role in partial elimination of mitochondria during these processes to facilitate metabolic reprogramming. Here we investigated if autophagy is needed for myocyte cell differentiation, to properly coordinate mitochondria and transcription factors regulating myocyte cell differentiation in muscle development. Methods. C2C12 cells were cultured in DMEM with 10% FBS and induced to differentiate by changing the medium to DMEM with 2% horse serum for 6d. Cells were transfected with Atg5 siRNA and

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infected with GFP-LC3 adenovirus. Cells were treated with 10nM Bafilomycin A1 or DMSO (vehicle control) for a 3hr interval daily for the first 3d. At the indicated times, cells were harvested and processed for western blot detection of LC3, MyoD, myogenin, alpha-actin, and Tom70. Total RNA was prepared from C2C12 cells. Reverse transcriptions were carried out with iScript cDNA Synthesis Kit (BIO-RAD) enzymes and random primers. The sequences of forward and reverse oligonucleotide primers, specific to PGC1-α and housekeeping genes, were designed. Real time quantitative PCR was performed in an iCycler 5 (BIO-RAD). The activities of the mitochondrial electron transport Complexes I-IV were measured using a Clarke oxygen electrode with a thermojacketed chamber. Fixed cells were immunostained with antibodies to alpha-actin. Results. We observed that during the differentiation of C2C12 myoblasts into myotubes, levels of LC3-II and abundance of GFP-LC3 puncta increased, consistent with induction of autophagy. Inhibition of autophagy with Atg5 siRNA or Bafilomycin A1 prevented myotube formation. This was associated with failure to upregulate the myogenic factors MyoD, myogenin and the mitochondrial import receptor Tom70. Furthermore, inhibition of autophagy with Atg5 siRNA attenuated expression of the mitochondrial biogenesis factor PGC1-α and oxygen consumption. These results suggest that inhibition of autophagy prevented mitochondrial maturation for energy production. Conclusions. Our studies demonstrate a role for mitochondrial biogenesis and activity during myoblast cell differentiation to myotube and suggest that autophagy is essential for myoblast differentiation. These findings may have relevance to cardiac stem cell differentiation.

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MYOGENIC DIFFERENTIATION OF HUMAN IPS CELLS USING GROWTH FACTORS AND SMALL MOLECULES IN DEFINED SERUM-FREE MEDIUM

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Reprogramming technology, iPS cells, and methods for inducing differentiation of tissue stem cells from pluripotent stem cells have demonstrated potential for treatment of human diseases such as muscular dystrophy, but there is still no effective protocol to differentiate human iPS cells into myogenic progenitor cells without transcription factors. To establish a protocol to induce myogenic differentiation of human iPS cells without using transcription factors, we tested methods to induce paraxial mesoderm from human iPS cells in a defined serum-free medium. To identify small molecules that induce paraxial mesodermal cells (PDGFRα+, Flk-1-) from embryoid bodies of E14 mouse ES cells in serum-free medium, we screened a library of 500 defined small molecules and identified five candidate compounds. Of the five small molecules, three induced paraxial mesodermal cells in mesodermal differentiated embryoid bodies in combination with Wnt3a and BMP4 from 201B7 human iPS cells. The induced paraxial mesodermal cells were isolated by FACS and underwent myogenic differentiation in cardiotoxin-injured muscle of NOD/SCID mice. These results suggest that growth factors and small molecules can induce myogenic progenitor cells from 201B7 iPS cells in a defined serum-free medium. We are currently testing a way to induce the myogenic differentiation of the induced paraxial mesodermal cells.

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MUSCLE DERIVED STEM CELL THERAPY FOR THE REGENERATION OF PELVIC MUSCLES

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Objectives: This study was designed to determine whether the injection of muscle-derived stem cells into the defected or injured female pelvic floors can regenerate their skeletal and smooth muscles and improve their functional properties in a rat model. We hypothesized that implantation of muscle derived stem cells into pelvic floors may improve their function, leading to a therapeutic approach for treatment of fecal incontinence. Methods: In this study, rats with sphincterotomy and repair were utilized as a model for anal sphincter dysfunction and fecal incontinence. The control group A underwent only sham operation. The study group B had sphincterotomy and repair of anal sphincters, followed by saline solution injections. The study group C underwent sphincterotomy and repair, followed by intra-sphincteric injections of muscle derived stem cells (MDSC). MDSC were isolated from gastrocnemius muscles of female rats, and then cultured and characterized. *In vivo* differentiation of MDSC was evaluated using immunofluorescence after injection of MDSC into nude rats in groups A, B, and C. The MDSC were labeled with PKH-26 before injections. At 4, 8, and 12 weeks after injections, these animals were studied for functional improvement by manometry and contractility testing, and for tissue histologic and morphometric analysis. Differentiation of implanted MDSC was assessed by immunofluorescence, using specific antibodies as markers of smooth muscle (smooth muscle actin, calponin, and smoothelin) and of skeletal muscle (MHC-II). MDSC were followed using their labeling marker PKH-26. Results: A significant decrease of muscle tissue was observed at the site of repair after sphincterotomy in groups B as compared to group A. However, in Groups C, histologic examination demonstrated new muscle fibers and morphometric analysis revealed a significantly greater muscle area fraction than in Group B. PKH-26-labeled implanted MDSC were detected in the anal sphincters. Differentiated muscle tissues stained positively for alpha smooth muscle actin and myosin heavy chain indicating the formation of smooth and skeletal muscle, respectively, at the MDSC injection sites. The MDSC injection sites were demonstrated by their label PKH-26. Functional studies showed improvement of anal sphincter function. Conclusions: This study demonstrates that MDSC injections improved muscle regeneration and function of the damaged anal sphincters. Regenerations of both skeletal muscle of the external sphincter and smooth muscle of the internal sphincter with MDSC may have potential application as a therapeutic approach to treating fecal incontinence.

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EPIGENETIC MEMORY ENHANCES MYOGENIC FATE OF INDUCED PLURIPOTENT STEM CELLS

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Muscular dystrophies (MDs) constitute a heterogeneous set of genetically inherited diseases, characterized by chronic degeneration of muscle fibres and, ultimately, by motility loss and cardiorespiratory failure. To date no regenerating strategies are yet present in the clinical practice. Cell replacement strategies could represent a valuable therapeutic alternative to regenerate damaged fibres and

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counteract muscle wastage. Murine and canine models of MD have shown improvements after systemic delivery and muscle engraftment of mesoangioblasts (MABs). MABs are somatic, vessel-associated stem cells, isolatable from murine, canine and human muscle biopsies. Notwithstanding their myogenic regenerative potential *in vivo*, MABs display a limited proliferation potential and rapidly undergo senescence in vitro. In order to expand MAB potency and self-renewal capacity, we generated MAB-derived induced pluripotent stem cells (MAB-iPSCs). MAB-iPSCs shared morphology, self-renewal and pluripotency features with fibroblast-derived iPSCs (f-iPSCs). Both MAB- and f-iPSCs could give rise to traceable parts of chimeric embryos after morula aggregation. Surprisingly, after subcutaneous injection in immunodeficient mice, MAB-iPSCs produced teratomas with a prominent presence of striated muscle tissue, unlike f-iPSCs. In addition, in vitro differentiation revealed a higher commitment of MAB-iPSCs toward MyHC+ myotubes and CD56+ myogenic progenitors. MAB-iPSC-derived progenitors showed efficient *in vivo* engraftment and fibre regeneration potential, after intramuscular injection into a murine model of severe MD. Furthermore, comparative tiling arrays on MAB- and f-iPSCs showed a source-biased methylation pattern on regions flanking many genes involved in myogenic differentiation. Recently, we generated human MAB-iPSCs and preliminary results are confirming a durable myogenic capability in vitro and *in vivo*.
Given their epigenetic memory and biased commitment, MAB-iPSCs could hence bridge the gap between pluripotent cells and MD regenerative therapies.

Endothelial Cells/Hemangioblasts

Poster Board Number: T-1161

HIGHLY EFFICIENT AND SIMULTANEOUS GENERATION OF HEMATOPOIETIC AND VASCULAR PROGENITORS FROM HUMAN INDUCED PLURIPOTENT STEM CELLS

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The derivation of engraftable vascular and hematopoietic stem cells from patient-specific human induced pluripotent stem cells (hiPSC) may have great clinical utility for the effective, long-term treatment of hemato-vascular disorders. However, recent studies have suggested that hiPSC do not produce hemato-endothelial progeny in a manner that is quantitatively and qualitatively comparable to human embryonic stem cells (hESC). There may be several etiologies for this limitation, including the quality of reprogramming achieved in fibroblast-iPSC (due to retention of somatic donor epigenetic memory), the method of hiPSC culture employed for maintaining pluripotency (e.g. on murine embryonic mouse fibroblasts (MEF) vs. feeder-free monolayer), and the inherent efficiency of the differentiation protocol (e.g., embryoid body vs. stromal co-culture-based). In these studies, we focused on optimizing our previously published hEB-based hemato-endothelial differentiation method for efficient hiPSC differentiation. We demonstrate that under modified feeder-free endothelial culture conditions, multipotent CD34+CD45+ hematopoietic progenitors emerged in mass quantities directly from adherent endothelial/stromal layers of differentiated human induced pluripotent stem cells (hiPSC), and in a manner similar to that which occurs *in vivo* from hemogenic endothelium. Using a modified human embryoid body (hEB) system, we simultaneously differentiated fibroblast-derived iPSC (fibroblast-iPSC) into both hematopoietic and vascular progenitor cells with comparable efficiency to hESC. Two previously described fibroblast-iPSC lines (IMR90-1 and IMR90-4) were differentiated in parallel with a hESC line (H9; WA09) into hematopoietic and vascular lineages with this method. HEBs from fibroblast-iPSC and hESC were evaluated during differentiation by FACS for the kinetics of hemato-entothelial marker expression (CD34, CD31, CD143, CD146, KDR, and CD133). These markers peaked at days 8-10 of hEB differentiation, thus this stage was further cultured onto endothelial growth medium (EGM2) and fibronectin-coated plates. In these conditions, clumps of hEB cells not only produced functional CD31+CD146+ vascular progenitors, but also directly differentiated into clusters of hematopoietic "cobblestones" with adherent RUNX1-expressing monolayers. After 3-6 days, floating cells emerged from these adherent endothelial cells that expressed high levels of CD34, CD45, and the hemangioblast marker CD143/ACE (BB9), and were enriched with superior frequencies of hematopoietic CFU. In this culture system Fibroblast-iPSC produced mass quantities of CD34+CD45+ cells and hematopoietic CFU with similar or higher frequencies than hESC. Further addition of hematopoietic cytokines (e.g. thrombopoietin, angiopoietin-1, erythropoietin, and IL-6) into EGM2 culture poised bulk quantities of differentiating cells into committed erythro-myeloid lineages. This novel and highly efficient hemato-endothelial differentiation system will be ideal for direct time course studies of hematopoietic genesis events (e.g. in vitro models of hemogenic endothelium) using time-lapse videography, or bulk FACS kinetic analyses of emerging hematopoietic progenitors from hemangioblast progenitors.

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BD™ PURECOAT™ ECM MIMETIC SURFACES: NOVEL SYNTHETIC, XENO-FREE, ANIMAL-FREE SURFACES FOR HUMAN ENDOTHELIAL COLONY FORMING CELL EXPANSION

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Endothelial colony forming cells (ECFC) have potential uses in regenerative medicine, such as cell therapies for cardiovascular disease. Expansion of these cells requires coating of the culture vessel with human or animal-derived extracellular matrix (ECM) protein which can introduce human and animal-derived pathogens in the culture. ECMs can be poorly defined and may have batch to batch variability. Moreover, self-coating can be time consuming and coated vessels have limited shelf life. For aforementioned reasons chemically defined extracellular matrix (ECM) systems are required. To support such research areas, we have developed two synthetic, animal-free (defined as free of any human or animal-origin component) peptide surfaces: BD PureCoat collagen I ECM mimetic and fibronectin ECM mimetic. Both of these scalable surfaces mimic native ligands for cell adhesion as demonstrated by attachment of specific integrin expressing cell lines. Peptide coated surfaces can be utilized to grow and expand ECFC, and are stable at room temperature. Human ECFC were cultured on these surfaces in ECFC supplemented EGM-2 medium for multiple passages. Cell attachment, morphology and growth over multiple passages were compared to natural extracellular matrix protein Collagen I or Fibronectin. Functionality of these cells was demonstrated in a tube formation assay frequently cited as a method to study angiogenesis. Post expansion ECFC were able to form capillary-like structures formed by endothelial progenitor cells. Our results suggest that these breakthrough next-generation cell culture environments can be used for the culture of endothelial colony forming cells where defined environment is desirable as well as in basic and applied research.

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EX VIVO DEVELOPMENT AND EXPANSION OF FUNCTIONAL ENDOTHELIAL PROGENITOR CELLS FROM HUMAN CORD BLOOD

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In human, angiogenesis is not only important for physiology and development, but also correlation with most cardiovascular diseases such as myocardial infraction. Recently, many reports further demonstrated that angiogenesis related closely with tumor development. Angiogenesis and vasculogenesis are regulated by the proliferation and differentiation of endothelial progenitor cells (EPCs). The current therapy for patients with cardiovascular diseases is surgical operation, which is with low successful rate and recovery rate. Now, EPC transplantation in cell therapy is developed actively, and has great potential for treatment of cardiovascular diseases. So, isolation and cryopreservation of EPCs from cord blood would be beneficial for future cell therapy. In this study, we hoped to develop a novel culture system for the development and expansion of EPCs. Firstly we used the systematic procedure with Factorial Design and Steepest ascent method to optimize the cytokine-containing medium that could facilitate EPC growth. Our results showed that EPCs could be isolated and established from cord blood mononuclear cells and could expand over 2 months in our developed EPC medium. In addition, we found that VEGF165,SCGF-α,b-FGF,SCF,IGF-1,FLT-3 ligand,HGF,EGF,IL-8, Hydrocotision, 2-phospho-L-Ascrobic acid and Heparin are necessary for EPC growth. In addition, after cryopreservation, thawed EPCs still maintained expansion ability. We also found that the expanded cells can form tube formation cultured in the Matrigel and had plentiful expression of CD34, CD31, CD144, CD105, CD309 and vWF by using flow cytometry analysis. In our laboratory, we have reported a culture system for EPC establishment from cord blood and for large production of functional EPCs. We believe that the results of this study must be beneficial to the basic research and future trials on clinical transplantation.

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FUNCTIONAL IMPROVEMENT AND NEUROPROTECTION PROVIDED BY HUMAN CEREBRAL ENDOTHELIAL CELLS INTRAVENOULY-TRANSPLANTED IN FOCAL ISCHEMIA RAT BRAIN

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Stable clonal cell line of human cerebral endothelial cell, named as HEN7, has been generated from human fetal telencephalon using a retroviral vector encoding v-myc gene. HEN7 cells were transplanted intravenously via tail vein in rat brain with photochemically induced focal cerebral ischemia, and the clinical effects for infarct size, edema volume, and clinical outcome were evaluated. FACS analysis showed HEN7 cells express the characteristics of cerebral endothelial cells. HEN7 showed positive immunoreactivity for vascular, stem cell, and tight junction proteins. HEN7 transplanted group showed reduced infarct lesion as identified by bioluminescence and X-gal staining located in the infarcted lesion border area. HEN7 transplanted group showed markedly reduced edema volume associated with MMP-9 expression reduction, and markedly increased nestin-positive cells around infarcted area. HEN7 transplanted group showed earlier recovery from the neurological deficit. Intravenously transplanted hCECs selectively migrated and integrated into cerebral ischemic lesion area and accelerate neurological functional recovery. This new hCEC-based cellular therapy is applicable for clinical trial in ischemic stroke patients.

Poster Board Number: T-1166

ROBUST NEUROGENIC AND VASCULOGENIC EXPRESSION ACCOMPANIES FUNCTIONAL EFFECTS OF HUMAN CEREBRAL ENDOTHELIAL CELL TRANSPLANTATION IN STROKE ANIMALS

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Background and aims: Stem cell therapy has emerged as an experimental treatment for stroke. Despite the reported functional recovery in transplanted stroke animals and limited clinical trials in stroke patients, a major gap in our knowledge is the mechanism of action underlying cell therapy. Here, we examine the fate differentiation of transplanted human cerebral endothelial cells (HEN6) in stroke animal model, using markers of neurogenesis and vasculogenesis, combined with stress and sensorimotor tests using the Rat Grimace Scale (RGC) and modified Neurogical Severity Score (mNSS). Methods: Ten-week old rats underwent a one-hour middle cerebral artery occlusion. Animals were randomly assigned to receive stereotaxic transplantation of vehicle, 1, 2, or 4 million HEN6 three hours after occlusion. Rats were euthanized at 7 days post-reperfusion for immunohistochemistry using antibodies against neuronal, vascular, and specific human transplanted cell marker. 2,3,5-triphenyltetrazolium chloride staining was conducted in alternate sections to reveal infarct volume. All animals were videotaped for 15 min at day 1, 3, and 7 for the RGS stress test. After recording, the sensorimotor function was assessed by mNSS. Results: Increased expression of host neuronal and vascular markers was detected in the stroke core, and closely adjacent to the transplanted cells. Some transplanted cells differentiated into a microvascular phenotype and juxtaposed to the host vasculature. Neurogenic and vasculogenic upregulation was more pronounced in animals that received the 4 million cell dose, but the other doses also exhibited both regenerative processes. Infarct volume in transplanted stroke animals was significantly lower than vehicle-infused stroke animals. The mNSS revealed significant improvement of sensorimotor functions in 4 million HEN6 in comparison with saline group ($p<0.05$). Interestingly, RGS revealed higher stress score in 4 million HEN6 than the other groups ($p<0.05$) Conclusions: We found a correlation between vasculogenesis and neurogenesis following transplantation of HEN6 suggesting a dual pronged regenerative process in stroke animals, which accompanied the recovery of sensorimotor functions. The increased stress behavior in transplanted stroke animals may be related to host immune response to the human xenograft. A better understanding of regenerative processes, as well as potential side effects will allow a critical assessment of the risk-to-benefit ratio of cell therapy in stroke.

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THE THERAPEUTIC POTENCY OF HUMAN INDUCED PLURIPOTENT STEM CELLS DERIVED ENDOTHELIAL PROGENITOR CELLS

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Introduction: Experimental and clinical studies have shown that endothelial progenitor cells (EPCs) can enhance angiogenesis in ischemic hindlimb muscles and myocardium. However, autologous EPCs transplantation is limited by the number and proliferative potential of the EPC isolated from patients' blood. Human induced pluripotent stem cell (hiPS) is a potential alternative cell source for EPC generation due to their autology, high power of proliferation and pluripotency. Methods: Donor specific Induced pluripotent stem cells were generated from their skin fibroblast in feeder free, serum free culture system and subsequently differentiated into EPCs (hiPS-EPCs) and its functions of in-vitro tube formation, migration ability and cytokine expression profiles, and in-vivo capacity to attenuate mice model of hind-limb ischemia were compared with the EPC differentiated from BM-MNC (BM-EPCs) and hESC (hESC-EPCs). Results: Differentiation of BM-EC from BM-MNC was only achieved in 1/6(17%) patients with coronary artery disease. Nevertheless, BM-EPCs, hESC-EPCs and hiPSC-EPCs exhibited typical cobblestone morphology, positive staining of vWF and the ability of DiI-Ac-LDL dye uptake, and Ulex europaeus lectin antigen expression. In-vitro functional assay demonstrated that hiPSC-ECs and hESC-ECs had similar capacity for tube formation and migration as BM-EPCs (all $p>0.05$). During hypoxia, increased expression of major angiogenic factors including epidermal growth factor, hepatocyte growth factor, vascular endothelial growth factor, placental growth factor and stromal derived factor-1 were observed in all EPCs cultures as compared with normoxia(all $p<0.05$). Compared with medium only, transplanting BM-ECs (n=8), hESC-ECs (n=8) or hiPSC-EPCs (n=9) into mice significantly attenuated severe hindlimb ischemia via enhancement of neovascularization. Conclusions: Our results demonstrate that hiPS-derived EPCs resemble normal human endothelial cells with similar phenotypes and angiogenic function but unlimited proliferation capacity. These findings suggest that hiPS-derived EPC can be used as patient specific cell source in therapeutic angiogenesis.

Poster Board Number: T-1168

ENDOTHELIAL PROGENITOR CELLS IN PERIPHERAL BLOOD OF CARDIAC CATHETERIZATION PERSONNEL

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The aim of the present study was to evaluate the rejuvenation capacity among cardiac catheterization personal occupationally exposed to ionizing radiation. Venous blood samples were obtained from 60 cardiac catheterization personals exposed to x-ray during fluoroscopy procedure at the National Heart Center in Embaba vs. 20 controls. Blood samples were assayed for total and differential blood count, micronucleus formation (FMN) plasma stromal growth factor (SDF-1) and cell phenotype of circulating endothelial progenitor cells (EPCs), whose surface markers were identified as the CD34, CD133 and kinase domain receptor (KDR). The individual annual collective dose ranged from 2.16 - 8.44 mSv/y as measured by thermoluminscent personal dosimeters (TLD). Results showed that SDF-1α and FMN were significantly higher among cardiac catheterization staff compared to controls. Similarly, EPCs: CD34, CD133, KDR were significantly increased among cardiac catheterization staff compared to controls. Smoking seemed to have a positive effect on the FMN and SDF-1 but a negative effect on EPCs. It is concluded that among cardiac catheterization staff, the numbers of circulating progenitor cells have increased and accordingly, increased capacity for tissue repair. In conclusion, the present work shows that working exposure to radiation, well within permissible levels, leaves a genetic mark on somatic DNA of the interventional cardiologist. However, exposure to ionizing radiation stimulates regenerative processes as indicated by the increase in EPCs and SDF-1. This regenerative process is decreased by smoking as evidenced by increased levels of SDF-1 and decreased levels of EPCs. The personnel who work in cardiac catheterization laboratories should carefully follow radiation protection procedures and should minimize radiation exposure to avoid possible genotoxic effects.

Poster Board Number: T-1169

THE HAEMOPOIETIC SUPPORTIVE MURINE BONE MARROW MS-5 MESENCHYMAL STROMAL CELL LINE HAS A NOVEL ROLE IN PROMOTING HUMAN VASCULOGENESIS AND ANGIOGENESIS

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The bone marrow contains specific microenvironmental stem cell niches which maintain haemopoiesis. CXCL12-expressing mesenchymal stromal cells are closely associated with the bone marrow sinusoidal endothelia, forming key elements of the haemopoietic stem cell niche, yet their ability to regulate endothelial function is not clearly defined. Since the murine nestin+ cell line, MS-5, provides a clonal surrogate bone marrow stromal niche capable of regulating both murine and human primitive haemopoietic stem/progenitor cell (HSC/HPC) fate *in vitro*, we hypothesised that MS-5 cells might also support new blood vessel formation and function. Here, for the first time, we demonstrate that this is indeed the case. Using proteome arrays, we identified HSC/HPC active angiogenic factors that are preferentially secreted by haemopoietic supportive nestin+ MS-5 cells, including CXCL12 (SDF-1), NOV(CCN3), HGF, Ang-1 and MCP-1(CCL2). Concentrating on CXCL-12, we confirmed its presence in MS-5 conditioned media and demonstrated that its antagonist in receptor binding, AMD-3100, which mobilises HSC/HPCs and endothelial progenitors from bone marrow, could significantly reduce MS-5 mediated human vasculogenesis *in vitro*, principally by regulating human endothelial cell migration. Thus, the clonal nestin+ MS-5 murine bone marrow stromal cell line not only promotes human haemopoiesis but also induces human vasculogenesis, with CXCL12 playing important roles in both processes. Funding: This work was supported by the National Health Service Blood and Transplant (NHSBT), EU Framework 7 Cascade project and the National Institute of Health Research (NIHR), UK, under its Programme Grant Scheme (RP-PG-0310-1001 and -1003). The views

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Poster Board Number: T-1170

WHISTLING IN THE DARK - UNDERSTANDING VASCULAR ANOMALIES AND IDENTIFYING VASCULOGENESIS AS PLAYING A MAJOR ROLE IN TUMOR FORMATION

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Shaghayegh Harbi - New York University Mentors: David J Kahler (Director of NYSCF Drug Discovery Laboratory); Paolo G Mignatti (Associate Professor New York University School of Medicine Departments of Cardiothoracic Surgery and Cell Biology); June K Wu (Assistant Professor Columbia University Department of Surgery) Though the most common tumor of infancy, little is known of the pathogenesis and etiology of infantile hemangiomas (IH). The working hypothesis is that the hemangioma derived stem cell (HemSC) is a vascular stem/progenitor cell whose proliferation is dysregulated but not fully transformed, that orchestrates hemangioma pathophysiology via a sophisticated activation of multiple signaling and regulatory networks. The overall goal of this investigation is to understand the process of hemangioma-genesis - defined as human vascular differentiation, followed by vascular regression and adipogenesis - by targeting the differences between the hemangioma-derived stem cells (HemSCs), hemangioma endothelial cells (HemECs), an intermediate cell population, and the heterogeneous cell population. Thus the specific aim is: a) to identify the intrinsic differences between HemSC and HemEC and compare HemSC and HemEC genetically and epigentically; b) analyze the "stemness" genes, paracrine/endocrine associated genes ("angiocrine"), and genes that regulate development, vasculogenesis and immunity; c) identify involvement of the signaling pathways at various stages of differentiation; d) provide new perspectives on the failure of anti-vasculogenic inhibitors currently in use. To achieve this goal, a multifaceted, direct target approach is proposed to study the regulation, relationship, and mechanisms that control differentiation and interaction of hemangioma-derived stem cells (HemSCs) and hemangioma endothelial cells (HemECs) by performing experimental studies to analyze: a) expression profiles to survey gene expression profiles and patterns between the four cell types by using microarray analysis followed by qRT-PCR; b) cell surface marker screen to isolate, screen, survey and characterize the various stages of differentiation of the four cell types by profiling human cell surface markers using flow cytometry and bioimaging of cell surface proteins. Specifically, to survey gene expression profiles between the four cell types (CD133+, CD133+CD31+, CD31+, heterogeneous population) of IH cells to further development of gene expression signatures, gene expression analysis was performed of different human cell samples using Agilent whole human genome oligo microarrays. In order to develop definitive and effective therapies of IH, a critical question is to determine which cells contribute to hemangiogenesis and disease progression. Does hemangiomagenesis follow a model where growth and progression of the tumor is driven by a small subpopulation of cells or does the bulk population of cells have tumorigenic potential? What is the interaction between environmental cues (such as hypoxia, hormones, immunity, stress, toxicity), progenitor cells, and associated signaling pathways (TGFβ, Notch, HIFs, Tyrosine kinases, MAPK/ERK, NFκβ, PI3K-AKT)? A better understanding of this interplay between clinical insight and manifestation, genetic and epigenetic analysis, cellular signaling and molecular markers, and hormonal and environmental regulation and alterations will help diagnose and potentially treat this vascular anomaly.

Poster Board Number: T-1171

A NOVEL ENDOTHELIAL MICRORNA MODULATING ANGIOGENIC RESPONSES

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MicroRNAs are a class of small RNAs that regulate target gene expression at the post-transcriptional level. Several microRNAs have been reported to control angiogenesis. We previously developed a novel embryonic stem (ES) cell differentiation system that exhibits vascular cell differentiation and early vascular development using vascular endothelial growth factor (VEGF) receptor-2 (VEGFR2/Flk1)-positive cells as common progenitors (Yamashita, Nature, 2000). With the use of this system, we have been elucidating various cellular and molecular mechanisms of vascular cell differentiation, such as enhancement of endothelial cell (EC) differentiation from Flk1+ cells with protein kinase A activation (Yamamizu, Blood, 2009) and arterial EC specification through direct interaction of Notch and beta-catenin signaling downstream of cAMP (Yurugi-Kobayashi, Arterioscler Thromb Vasc Biol, 2006; Yamamizu, J Cell Biol, 2010). In this study, to elucidate roles of microRNAs in EC differentiation and vascular formation, we screened microRNAs specifically expressed during EC differentiation with the use of our ES cell differentiation system. Then, we identified a specific microRNA (mir-X) that is expressed in vascular ECs from ES cells, more predominantly in arterial ECs than venous ECs. Mir-X is expressed also in ECs of the dorsal aorta in the mouse embryo and in human EC lines. Overexpression of mir-X in human EC lines showed an inhibitory effect on angiogenic responses such as EC migration or tube formation. *In vivo* functions and target molecules of mir-X are currently investigated. Mir-X is, thus, supposed to be an endogenous regulator of angiogenesis, suggesting its great possibility for clinical application in the treatment of cancer or ischemic diseases.

Poster Board Number: T-1172

EFFECT OF MICROPATTERNED PEPTIDES ON ENDOTHELIAL PROGENITOR CELL ADHESION AND EXPANSION

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Half of the small-diameter vascular prostheses used in bypass and replacement surgeries become occluded within 5 years. The intimal hyperplasia that leads to graft failure could be prevented if the luminal prosthetic surfaces were engineered to recruit circulating progenitor cells capable of generating a healthy endothelial cell lining. We have previously described a method to increase the endothelialisation rates of prosthetic materials *in vitro* by micropatterning two peptides derived from the integrin-binding regions of fibronectin. The objective of the current work was to examine the mechanism by which these micropatterns affect endothelial progenitor cell adhesion and proliferation. Glass surfaces were

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functionalized with 10.1 ± 0.1 μm diameter CGRGDS peptide spots covering 20% or 50% of the surface, with CWQPPRARI covering the remaining surface. Endothelial progenitor cell-derived CD31+ cultures were expanded from adult human saphenous veins. Compared to untreated surfaces, the micropatterned surfaces led to a 2-fold increase in the maximum cell surface area after cell adhesion and spreading. Fluorophore-tagged RGD micropatterns revealed that cells preferentially extended pseudopods on the RGD spots. In addition, 86 ± 17% of the focal adhesions were located on the RGD spots, with a high frequency near spot edges. The concentration of focal adhesions on the RGD spots was independent of the spot size, but increased at higher spot densities. Conversely, cells on untreated or gelatin-coated surfaces remained rounded with few focal adhesions. These results suggest that the RGD spot density impacts focal adhesion formation and hence cell spreading kinetics. We are now investigating the effect of peptide micropatterns on umbilical cord blood-derived endothelial progenitor cell fate.

Poster Board Number: T-1173

FOUR-YEAR OUTCOME AFTER INTRAMUSCULAR TRANSPLANTATION OF GCSF-MOBILIZED CD34+ CELLS IN NO-OPTION PATIENTS WITH CRITICAL LIMB ISCHEMIA

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Background: Prognosis of chronic critical limb ischemia (CLI) patients, in whom conventional revascularization is neither successful nor indicated, is extremely poor, and the development of novel strategy for blood flow recovery is urgently needed for such intractable disease. Therapeutic potential of endothelial progenitor cells (EPCs) has been established by a number of preclinical studies for hindlimb, myocardial and cerebral ischemia. Our phase I/IIa clinical trial revealed that intramuscular transplantation of autologous, granulocyte colony stimulating factor (GCSF)-mobilized CD34+ cells, an EPC-enriched fraction, was safe, feasible and effective at week 4 and 12 post cellular therapy in 17 patients with CLI in whom conventional revascularization was not indicated. However, long-term outcome of the stem/progenitor cell therapy has never been reported. Methods: No-option patients with CLI by atherosclerotic peripheral arterial disease (PAD) (N=5) or Buerger's disease (N=12) underwent leukoapheresis following 5-day subcutaneous infusion of GCSF (5-10 μg/kg/day). CD34+ cells were isolated from the apheresis product by a magnetic sorting system, CliniMACS®, then intramuscularly transplanted into 40 sites of ischemic lower limbs. Incidence of major clinical events including death, major amputation, unplanned minor amputation and other major cardiovascular events, and physiological parameters of limb ischemia were evaluated at year 1, 2, 3 and 4 post CD34+ cell transplantation. Results: No patients died by year 1, whereas 3 patients with PAD died by year 3 and 1 patient with Buerger's disease died by year 4 due to cardiac complications. No patients underwent major amputation, whereas 1 patient with Buerger's disease underwent unplanned minor amputation by year 2. Non-CLI status (Rutherford's category ≤ 3) was achieved in 82% at year 1, 88% at year 2, 92% at year 3 and 85% at year 4 in all patients. Significant improvement of toe brachial pressure index (TBPI) was sustained up to year 4 vs baseline (0.44±0.28 vs 0.21±0.17, P=0.004) and that of transcutaneous partial oxygen pressure (TcPO2) was kept up to year 3 (52.1±12.5 vs 23.8±21.4, P=0.02). Ulcer size (5.7±18.0 vs 38.0±21.3 mm, P<0.0001), total walking distance (850.2±269.2 vs 436.2±356.2 m, P<0.0001), pain-free walking distance (895.3±219.4 vs 233.0±320.2 m, P<0.0001) and Wong Baker FACES pain rating scale (0.5±0.5 vs 2.4±0.7, P<0.0001), which were examined until year 1, significantly improved at year 1 compared with baseline. Subgroup analysis revealed the similar outcome in patients with Buerger's disease. Conclusions: Favorable clinical outcomes as well as physiological evidences strongly indicate the long-term benefit of GCSF-mobilized CD34+ cell transplantation for retrieval from CLI, especially in patients with Buerger's disease. These promising results encourage a phase III, randomized clinical trial in the near future.

Lung Cells

Poster Board Number: T-1174

ALDEHYDE DEHYDROGENASE 2 IS ESSENTIAL FOR SELF-RENEWAL OF STEM/PROGENITOR CELLS OF THE PROXIMAL AIRWAY EPITHELIUM.

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Basal cells of the airways have been found to repair the surface epithelium. We identified a multipotent stem/progenitor epithelial cell population in the submucosal gland duct of the human and murine airway, which is capable of regenerating the submucosal glands and the surface epithelium overlying the submucosal glands. Microarray analysis showed aldehyde dehydrogenase genes ALDH1A1, ALDH2 and ALDH3A1 are highly expressed within both basal and submucosal gland duct cell populations. Work from many groups has shown that cell populations sorted from several different tissues based on their high ALDH activity are enriched for stem/progenitor cells. However, the role of ALDH in airway progenitor/stem cells has not yet been elucidated and it is not clear which isoforms might be important for self-renewal and differentiation. The airways are in contact with the environment and are directly exposed to air pollution and cigarette smoke, which trigger the formation of reactive oxygen species (ROS). ROS cause oxidative breakdown of cell membrane lipids followed by the accumulation of reactive aldehydes. Reactive aldehydes cause apoptotic cell death and therefore airway cells in general, and airway progenitors in particular, need a high level of aldehyde dehydrogenases in order to protect themselves from these harmful reactive aldehydes. We used the Aldefluor® fluorescent reagent system to isolate the cells that express high levels of ALDH within the airway basal and submucosal gland duct cell populations from both mouse and human. We found that only ALDH high expressing (ALDH-hi) basal cells and submucosal gland duct cells were able to self-renew and form spheres in matrigel from either human or mouse airway epithelium. We performed immunostaining of the airways for these ALDH isoforms and found ALDH2 more highly expressed in basal and duct cells than in differentiated cells. The reverse was true for ALDH1A1, with expression being higher in differentiated cells. ALDH3A1 staining was faintly seen in basal cells and was bright in the myoepithelial cells of the submucosal glands. In order to functionally examine the role of ALDH2 in basal and duct cell self-renewal and differentiation, we treated sorted cells in the sphere assay with the ALDH2 inhibitor, Diadzin. Diadzin treatment resulted in forma-

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tion of smaller and fewer spheres without affecting differentiation of the spheres, indicating the importance of ALDH2 for basal and duct progenitor cell self-renewal. ALDH2 has been shown to inhibit acetaldehyde-induced ROS production. We therefore hypothesized that ALDH2 and ROS levels within airway progenitor cells would be inversely proportional and be related to the self-renewal potential of the cells. We therefore used DFA and MitoSOX to sort ROS-high versus ROS-low basal cells and submucosal gland duct cells. Only ROS-low basal or duct cells had the capacity to self-renew and form spheres. The ALDH-hi and ROS-low populations were found to markedly overlap and treating this sphere-forming population with hydrogen peroxide inhibited sphere formation, indicating that tight regulation of ROS levels is required for self-renewal of airway stem/progenitor cells and that ALDH2 might be involved in this regulation. ALDH2 is necessary for self-renewal of airway epithelial progenitor/stem cells. We speculate that adult stem cells in the airway may be afforded the ability to survive injury from environmental pollutants, such as smoke, by the expression of relatively high levels of ALDH2.

Poster Board Number: T-1175

SEQUENCE-SPECIFIC GENETIC CORRECTION OF IPS CELLS DERIVED FROM PATIENTS WITH INHERITED LUNG DISEASE

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Cellular transplantation of lung stem/progenitor cells represents a potential therapeutic approach for a variety of inherited monogenic lung diseases. Crucial to the success of such a therapeutic strategy is that the transplanted cells and their progeny are corrected for the disease-causing mutation and that the transplanted cells do not elicit an immune response in the recipient. In order to satisfy these criteria, we are pursuing a patient-specific approach in which, starting with skin or blood cells from patients with inherited lung disorders, autologous induced pluripotent stem (iPS) cells are first derived. Utilizing site-specific homology-directed repair, the disease-causing mutation is corrected in the endogenous, chromosomal DNA sequence. Finally, a directed differentiation approach is employed to obtain highly purified populations of the relevant lung stem/progenitor cells from the corrected iPS cells for purposes of transplantation. We have initially employed this approach to generate corrected, autologous iPS cells for patients with Cystic Fibrosis (CF). Starting with CF patient fibroblasts, we have derived and extensively characterized iPS cell lines, confirming their pluripotency and normal karyotype. We then utilized Zinc Finger Nucleases, designed to target the endogenous CFTR gene, to mediate correction of the inherited genetic mutation in this locus via homology directed repair. We have demonstrated that the corrected CF iPS cells, when induced to differentiate *in vitro*, express the corrected CFTR gene. Importantly, we observed an exquisitely sensitive, homology-dependent specificity of targeting one CFTR allele vs. the other. This allele-specific targeting offers the potential for preferential targeting of ZFN-mediated correction to dominant mutant alleles.

Poster Board Number: T-1176

THE WNT/B-CATENIN PATHWAY REGULATES THE SELF-RENEWAL AND DIFFERENTIATION OF TRACHEAL BASAL CELLS

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Basal cells (BCs) in the mammalian airway are thought to include airway stem cells, which maintain and repair the pseudostratified epithelium of the conducting airways. Previous studies have shown that the Wnt/β-catenin signaling pathway regulates the cell-fate decision and differentiation of mouse tracheal BCs. However, its role in the self-renewal of tracheal BCs has not been studied, partly because previous studies employed methods which do not facilitate the analysis of self-renewal of tracheal stem cells. Using a tracheosphere culture system with an air-liquid-interface, we show that the Wnt/β-catenin signaling pathway regulates the self-renewal as well as differentiation of tracheal BCs. We find that inhibition of Wnt/β-catenin signaling by IWP2 or DKK-1 greatly decreased primary tracheosphere formation and the luminal cell population. In the subsequent passage, IWP2 or DKK-1 treatment in primary culture decreased secondary tracheosphere formation without further treatment. On the other hand, activation of Wnt/β-catenin signaling by GSK-3β inhibitor increased the luminal cell population in primary culture. Taken together, these data show that Wnt/β-catenin signaling pathway regulates the self-renewal of tracheal BCs.

Poster Board Number: T-1177

RESYNCHRONIZATION OF MITOSIS IN NORMAL AIRWAY EPITHELIAL PROGENITOR POPULATIONS IS COINCIDENT WITH FLUCTUATIONS IN TGF-B1 SECRETION

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The proliferative characteristics of airway epithelial progenitors are likely to be important for restoring lung homeostasis in diseases of ongoing epithelial injury, such as asthma. To that end, we recently showed that normal *in vitro* tracheobronchial epithelial progenitor populations are characterized by relatively synchronous progression through the cell cycle while asthmatic progenitor populations proliferate with a more even distribution of cells in each cell cycle phase. Further, this asthmatic "mitotic dyssynchrony" results in sustained elevation in TGF-β1 secretion. Because TGF-β1 troughs and peaks regulate mitotic synchrony in non-airway epithelia, we suspect that sustained asthmatic secretion of TGF-β1 implies a defect in TGF-β1 induced mitotic regulatory signaling. As a first step toward identifying this defect, we hypothesized that resynchronization of mitotic dyssynchrony in proliferating normal airway epithelial progenitor populations is coincident with a trough and peak in TGF-β1 secretion. Mitotic dyssynchrony was induced in parallel cultures of normal proliferating tracheobronchial epithelial cells via transient serum starvation (for 12 hours) in a staggered fashion. Aliquots of each were reserved as controls prior to mixing the cultures. Cultures were continuously exposed to bromodeoxyuridine (BrdU). Cells and media were collected at 0, 12, 18, 24, 30, 42 and 48 hours. Mitotic phase was analyzed by flow cytometry for 7-AAD DNA staining in BrdU+ cells. Supernatant TGF-β1 was analyzed by ELISA. The mixed cultures were mitotically dyssynchronous at all time points through 30 hours to a similar degree (e.g., G1/S/G2,M

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for 30 hours: 54/20/26%), began to spontaneously resynchronize at 42 hours (69/14/17%), and was fully synchronous by 48 hours (2/89/9%). The control cultures remained synchronous throughout, but out of phase with each other and the mixed cultures. Percentage of baseline TGF-β1 (measured at 0 hours) was calculated for 18 (-9.4%), 24 (-10.3%), 42 (+2.7%) and 48 (+1.8%) hours in the mixed culture. In contrast, the control cultures' TGF-β1 percentage of baseline remained low at 24 hours (-10.5,-8.6%) and 48 hours (-1.8,-6.5%). We developed a novel *in vitro* model of induced mitotic dyssynchrony and spontaneous mitotic resynchronization in normal tracheobronchial epithelial progenitor cells that will be useful to dissect mitotic regulatory signaling. Importantly, our data show that this mitotic dyssynchrony and resynchronization occur in concert with a trough and peak in TGF-β1 secretion. This model permits study of TGF-β1 regulation of normal airway epithelial progenitor mitotic synchrony, laying the foundation for experiments to determine the defect(s) that underlie asthmatic mitotic dyssynchrony.

Poster Board Number: T-1178

TARGETED CCSP$^+$ SCA1$^+$ MURINE BONE MARROW CELL THERAPY RESTORES CFTR AND IMPROVES LUNG FUNCTION

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Cellular therapy is a promising approach for treatment of lung disease such as cystic fibrosis. However, previous studies reported low engraftment while the function of grafted cells remained unclear. Conditions to increase airway engraftment and therefore cystic fibrosis trans-membrane receptor (CFTR) expression have not been described. We previously showed better bone marrow cell (BMC) retention in the lung for trans-tracheal delivery versus intravenous injection and optimized other delivery parameters leading to increase retention efficiency and greater long term engraftment. We also identified a subpopulation of BMC expressing clara-cell secretory protein (Ccsp) which can proliferate and differentiate into lung epithelium. Here, we isolated an even smaller population of BMC expressing Ccsp and stem cell antigen-1 (Sca1). We expanded this population by approximately 10-fold in culture with mouse embryonic fibroblast (MEF) feeder layer for 15 to 20 days before sorting out GFP positive cells and using them for delivery to injured lung. Expanded Ccsp+Sca1+ cells had ~5-fold greater retention efficiency in mice lungs, as detected by real-time PCR of the Y chromosome, when compared to unsorted 7-day cultured BMC. CFTR appeared to be restored in lungs of mice knockout for CFTR treated with wild-type cells and some cells were localized in the airway. Engrafted cells also appeared to have beneficial effects in lung function by maintaining ceramide levels which decreased with age in CFTR knockout mice not receiving any treatment. Airway surface liquid (ASL) thickness was also maintained to wild-type levels in CFTR knockout mice treated with wild-type cell. We propose that greater BMC engraftment in the lung will increase CFTR expression, possibly leading chloride transport restoration, and have other beneficial paracrine effects, thus improving lung function in cystic fibrosis disease.

Epidermal Cells

Poster Board Number: T-2001

MOVING TARGETS: PHENOTYPIC INSTABILITY OF A TET-OFF-SV40 TAG IMMORTALIZED CELL LINE ISOLATED FROM TRANSGENIC MOUSE ADULT SKIN TISSUE

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Introduction: From the double transgenic mice expressing SV40Tag driven by the tetracycline-transactivator (tTA, tet-off), a mouse cell line from the skin tissue was isolated and established in culture. That is unusual because conventional mouse skin cells are normally difficult to grow in vitro. Material and Methods: cell culture, apoptotic test, transgenic insertion of vectors coding for various cytokine, FACS analysis, ELISA assay, histology, confocal microscopy, RT-PCR assay, immune-incompetent mice. Results: The partial characterization of this mouse cell line demonstrates properties mimicking mouse ectodermal progenitor cell or even earlier precursor. The cells become apoptotic and necrotic in responding to doxycycline and TNF. Differentiation of these progenitor/stem cells does not occur automatically. It requires multiple factors and extensive manipulation in vitro and *in vivo*. Applying such approaches, we have isolated several cell clones. They are characterized to be down-stream keratinocytic precursors. The cell line is not stable, upon prolong culture, their phenotypes have shifted to more mature progeny. Conclusion: The stage of differentiation from ectodermal progenitor to keratinocyte could be assigned to be seven-plus stages. The doxycycline-regulated SV40Tag expressing, stage-specific cell lines isolated from skin and the derived cytokine secreting clones might be useful for multiple purposes such as functional genomic analysis, skin toxicology test, the mechanism of skin tumor progression, studying immune responses after skin stem cell transplantation.

Poster Board Number: T-2002

TRACING FOLLICULAR STEM CELLS IN ADULT MOUSE UNDER WOUND CONDITION

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Stem cells are multipotent and capable of self-replicating and differentiating into several cell lineages. In skin, the keratin 15 (K15)-positive cells in hair follicle bulge are stem cells, which can differentiate into cells of hair follicles and sebaceous glands. To precisely trace the stem cells, a R26 dual-fluorescence reporter mouse, activated by Cre-mediated DNA recombination, had been generated for labeling target cells with chromatin EGFP and membrane mCherry fluorecence(R26H2B-EGFP-mCHerry-GPI, abbreviated as R26GM). This reporter mouse had been an excellent tool to trace individual cells by their sharp nuclear green fluorescence and membrane red fluorescence. For identifying and tracing the stem cells in skin appendage, we crossed K15-CrePR mouse with R26GM mouse. The back skin of the inducible transgenic mouse (K15-CrePR-R26GM) was applied with 1% topical RU486 for 5 consecutive days and then skin tissues were sampled at different time points. We have traced the stem cells by monitoring the fluorescent cells in the hair cycling. The descendants of hair follicle stem cells, labeled with dual-fluorescence, migrated out of bulge during the anagen phase.

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Of note, the k15-positive stem cells can differentiate to a portion of interfollicular epidermis under wound condition. For clearly tracing the hair follicle stem cells and their descendants in different wound stages, a 1-cm full-thickness longitudinal incision was made on the back of the adult transgenic mouse (K15-CrePR-R26GM). We found the labeled cells migrated from bulge towards the interfollicular epidermis in the early phase. In later stage, the number of labeled cells decreased in the interfollicular epidermis.

Poster Board Number: T-2003

THE HISTONE METHYLTRANSFERASE SETD8 IS REQUIRED FOR SURVIVAL OF MOUSE EPIDERMAL STEM CELLS

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Epigenetic modifications, such as histone methylation play an important role in chromatin structure and promoter activity, and have been implicated in a wide range of biological processes including development, reprogramming, aging and cancer but our knowledge is still limited when it comes to the importance of histone methylation in multipotent stem cells of adult mammalian tissues. In this study we conditionally deleted Setd8/PR-Set7/KMT5a, the sole enzyme to catalyse the formation of mono-methylated histone 4 at lysine 20 (H4K20me1) in mouse skin. We targeted deletion of Setd8 to undifferentiated layers of the mouse epidermis and found both proliferation and differentiation processes impaired in embryonic and adult skin. We provide evidence that the long-lived epidermal progenitor cells are lost in the absence of Setd8, leading to a complete loss of sebaceous glands and interfollicular epidermis. We further show that Setd8 is a transcriptional target of c-Myc and an essential mediator of Myc-induced epidermal differentiation. Deletion of Setd8 in c-Myc over-expressing skin blocks cellular proliferation and differentiation and causes cell death instead. The increase in apoptosis in skin when Setd8 has been knocked-out can be explained by our discovery that p63, an essential transcription factor for epidermal commitment, is lost. In contrast, the p63 homologue p53 is gained upon removal of Setd8 in skin. Then, we show that both over-expression of p63 and repression of p53 can at least in part rescue the Setd8-induced epidermal phenotype. In this study we demonstrate for the first time that Setd8 is required for normal tissue homeostasis, *in vivo* and that Setd8 is an inhibitor of apoptosis in skin and its activity is essential for proper deposition of histone modifications at H4, epidermal stem cell survival as well as proliferation and differentiation. Finally, conditionally deletion of Setd8 in bulge stem cells of the hair follicle did not cause any hair or skin phenotype, indicating that stem cell populations located in the hair follicle bulge or interfollicular epidermis and sebaceous glands are distinct from each other.

Poster Board Number: T-2004

ANALYZING SKIN DEVELOPMENT AND HOMEOSTASIS WITH A TCF3 REPORTER KNOCK-IN MOUSE

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Tcf3 (also known as Tcf7l1) is a member of the Lef/Tcf family of transcription factors, which are best known as downstream cofactors of β-catenin in the canonical Wnt signaling pathway. Lef/Tcf proteins have been implicated in stem cell function in a variety of tissues, and Tcf3 in particular is a key regulator of differentiation/self-renewal balance in embryonic stem cells. Tcf3 expression has also been demonstrated in local stem and progenitor cells in mouse skin, where it plays an important role in tissue homeostasis and wound repair. However, the exact role played by the various Tcf3-expressing populations during skin development, renewal, and repair is unclear, and in general Tcf3 remains less well studied than other members of the Lef/Tcf family, especially its closely related paralogue Tcf4 (Tcf7l2). To allow a thorough analysis of the functions of Tcf3-expressing progenitor cells in skin and other tissues, we have created a novel Tcf3 reporter knock-in mouse. The *Tcf3-2A-eGFP-2A-CreERT2* (or "*Tcf3 GC*") mouse contains a eGFP fluorescent marker and tamoxifen-inducible Cre recombinase genes appended to the 3′ end of the native Tcf3 open reading frame using "self-cleaving" 2A peptides, generating three separate peptides from a single ORF. The resulting mouse is expected to express eGFP (for live cell sorting) and CreERT2 (to allow Cre/loxP-based lineage tracing) in all Tcf3-expressing cells. The targeting scheme was designed to preserve a functional Tcf3 ORF in order to allow the generation of homozygous knock-in mice. We generated chimeric knock-in mice using standard gene targeting techniques and verified that the knock-in allele was transmitted to progeny in the germline. Initial crosses yielded viable heterozygous (*Tcf3 GC/+*) and homozygous (*Tcf3 GC/GC*) knock-in mice without obvious abnormalities, demonstrating that the targeted allele retains normal function (Tcf3-deficient mice die during early development). In preliminary experiments with *Tcf3 GC/+; ROSA26 lacZ/+* mice, we observed Cre recombinase activity in the outer root sheath and bulge of the hair follicle, both known sites of Tcf3 expression. We are currently undertaking further experiments to assess GFP expression and Cre dose response in GC/+ and GC/GC mice, and we will then proceed with lineage-tracing experiments to assess the function of Tcf3-expressing cells during development and homeostasis.

Poster Board Number: T-2005

SUCCESSFUL GRAFTING OF AUTOLOGOUS TISSUE-ENGINEERED SKIN SUBSTITUTES ON BURN PATIENTS: PRESERVATION OF STEM CELLS AND MINIMAL CONTRACTION

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To adequately and permanently restore organ function after grafting, tissue-engineered skin substitute (TES) must ultimately contain and preserve functional epithelial stem cells. Another consideration is to maximize the structural stability of skin substitutes considering that the main sequalae of patients surviving major burn injuries result from contractures and hypertrophic scars. The present study was designed to assess stem cell preservation as well as the contractile behavior of the tissue-engineered skin (TES) made by the self-assembly approach, where keratinocytes are cultured on tissue-engineered dermis comprised of fibroblasts and the endogenous extracellular matrix they synthetized and organized. A subset of basal cells exhibiting the stem-cell associated slow-cycling property were identified within the epidermis of the TES cultivated *in vitro* using 5-bromo-2′-deoxyuridine (BrdU) labeling. A high proportion of these slow-cycling cells also expressed keratin 19 (K19). These stem cells were preserved in the epidermal basal layer of TES for several weeks of culture. The structural stability of the TES was evaluated *in vitro* at the end of the production (17 days of epidermal cell culture). The TES were detached from their anchor-

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ing, deposited on a soft substrate, and contraction was monitored over one week. Most of the contraction occurred within the first 12 hours following deposition on the substrate. The average contraction was only 24±4% for TES containing terminally differentiated epidermal layers. TES were then used as autologous full-thickness grafts for third-degree burn wound coverage or hypertrophic scar revision of severely burned patients. Before grafting, TES were detached from their anchoring and deposited on the soft substrate for at least 6 hours. One week after grafting, a complete graft take was obtained and no significant contraction was observed. Histological and immunofluorescence analyses revealed that the TES presented a well-organized epidermis and that stem cells were settled and maintained in the basal layer 21 days after grafting as identified by K19-labeling. Transmission electron microscopy observations confirmed the abundance of extracellular matrix elements in the dermis of grafted TES and the presence of a complete basement membrane with numerous hemidesmosomes indicating cohesion between the dermis and epidermis. TES promoted a particularly good healing and suppleness. The integrity of the transplanted TES persisted over time (2 to 6 years follow-up) with no defect in epidermal regeneration, and no significant contracture. We conclude that the TES produced by the self-assembly approach is a promising skin substitute for the closure of full-thickness skin injury because of its functional characteristics: in addition to preserve stem cells, it undergoes minimal contraction after grafting.

Poster Board Number: T-2006

NEURAL STEM CELL DERIVED CONDITIONED MEDIUM SUPPRESSES MELANOGENESIS THROUGH INHIBITION OF WNT/B-CATENIN SIGNALING PATHWAY

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Skin pigmentation by melanin serves many valuable functions such as photoprotection of the body from ultraviolet (UV) light, but production of excessive melanin triggers hyperpigmentation in the skin. Melanogenesis has been involved in complex regulatory control by various extrinsic and intrinsic factors that may be produced by neighboring cells in the skin or by the environments. Melanin is synthesized from tyrosine via an enzymatic cascade that is controlled by three important proteins such as tyrosinase, tyrosine-related protein 1 (TRP1) and dopachrome tautomerase (DCT). The most important microphtalmia-associated transcription factor (MITF) in the expression of tyrosinase, TRP1 and DCT genes is known as a master regulator of pigmentation as well as a target for the Wnt signaling pathway during the melanocyte differentiation program. Previously, the conditioned medium (CM) from many different types of adult stem cells such as adipose-derived stem cells has been identified its ability for the inhibition of melanin production. However, the neural stem cell-derived CM (NSC-CM) in the control of melanogenesis has not been investigated. In this study, we elucidated the effects and the mechanisms of the NSC-CM for melanin inhibition in vitro and *in vivo*. The melanin content and tyrosinase activity in B16 melanoma cells that were treated with NSC-CM were dramatically decreased. The key enzymes, which are tyrosinase, TRP1, DCT and MITF, were also robustly decreased in our real-time RT-PCR, western blot and immunofluorescence assays in vitro. In addition, the melanin content in C57/BL-6J mice that were treated with NSC-CM every once a day for 10 days was decreased when it was measured by Mexameter. The key enzymes were significantly decreased in our immunohistochemistry and immunofluorescence assays *in vivo*. Interestingly, we found that the NSC-CM antagonized the canonical Wnt pathway by decreasing of β-catenin accumulation in the nucleus, which was resulted from the increased Wnt inhibitors and the reduced co-receptors. Therefore, these results show that the NSC-CM could inhibit melanin synthesis through the down-regulation of MITF and its downstream targets, which is mediated by Wnt/β-catenin signaling pathway.

Poster Board Number: T-2007

THE OUTCOME OF SEBACEOUS C-MYC ACTIVITY IS DICTATED BY AN ANDROGEN RECEPTOR / P53 DIFFERENTIATION AND PROLIFERATION AXIS

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c-Myc (Myc) is the classic oncogene, however the paradox of Myc-induced epidermal differentiation is well documented. Analysis of mouse sebaceous glands (SG) revealed Myc expression coincided with Androgen Receptor (AR) expression, and in studies using K14-MycER transgenic mice (Myc mice), we found following Myc activation, the AR functioned to promote differentiation and inhibit proliferation. p53 activated in response to high Myc activity and inhibited AR signalling, thereby promoting only Myc's default proliferative function. Strengthening AR signalling with testosterone, inhibited p53 activity, forming a bidirectional AR/p53 differentiation axis by mutual antagonism. Analysis of human sebaceous neoplasms revealed AR expression correlated with increased differentiation, while p53 correlated with reduced differentiation. This bidirectional AR/p53 axis explains the paradox of how Myc can trigger both SG differentiation and proliferation in different contexts.

Poster Board Number: T-2008

THE DERMAL NICHE OF MURINE EPIDERMAL STEM CELLS IS AN UNEXPECTED PLASTIC TISSUE

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Mammalian dermis provides a niche for epidermal stem cell during tissue morphogenesis and maintenance. Dermal fibroblasts are the major component of this microenvironment and critical mediators of epithelial-mesenchymal interactions. Hair follicle formation for example is dependent on specialised dermal cells found in the dermal papilla and dermal sheath. Postnatal skin does not normally give rise to new hair follicle, but epidermal activation of beta-catenin in transgenic mice can induce ectopic hair follicles (EF). These EFs stimulate dermal fibroblasts to form ectopic dermal papillae indicating reciprocal signalling between epidermal stem cells and their niche. Here we show that epidermal Wnt/beta-catenin can stimulate the fibroblasts of the adult dermal niche to proliferate and remodel the extracellular matrix to an extant similar to neonatal dermis. Also, we identified a number of candidate fibroblast mitogens that were upregulated in keratinocytes in response to beta-catenin stabilisation, such as members of the family of the transforming growth factor beta (TGF-beta). This suggested a cross-

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talk between TGF-beta signalling and epidermal Wnt/beta-catenin signalling in this context. To examine the possible functional interaction between both pathways, we used a chemical inhibitor to modulate TGF-beta signalling during beta-catenin induced EF formation. TGF-beta inhibition caused epidermal cell proliferation in wild-type and transgenic mice leading to abnormal differentiation of the epidermal compartments. Mice developed dermal thickening indicating increased collagen deposition and increased proliferation of dermal fibroblasts. Overall, our results show that adult murine dermis is an unexpected plastic tissue.

Cardiac Cells

Poster Board Number: T-2009

REPLICATIVE SENESCENCE OF HUMAN CARDIAC STEM CELLS

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Recently cardiac stem cells (CSC) identified in human myocardium niche, these stem cells one of the potential sources for regeneration of human myocardium. CSCs have multipotent capability as transdifferentiation into cardiomyocyte, endothelial, and smooth muscle linage cells. However, CSCs have a limited lifespan in vitro as somatic, and other adult stem cells. Accumulating cell division, CSCs enter senescence, which observed enlargement of cytoplasm and a stop of in vitro amplification. Recently studies have indicated that CSCs exhibit reduced differentiation potential upon prolonged *in vitro* culture. Furthermore, senescence of CSC might limit their therapeutic applications. Thus, analysis of *in vitro* senescence in CSC is important to cell therapy based on stem cell for cardiovascular disease. However, molecular mechanisms of *in vitro* senescence of CSC are still poorly understood. Clearly, cellular senescence is a very complex process and the sequence of its molecular events is thus far unknown. Thus, we examined change of MAPK expression after CSC senescence and whether CSC senescence prevents used by MAPK regulation. In this study, using human CSC isolated from infant myocardium using by a c-kit antibody. Senescence human CSC (S-hCSC) used passage numbers of 11 to 14 and young human CSC (control) used passage number of 3 to 5. To characterize human CSC senescence, we examined senescence associated β-galactosidase activity (X-gal stain), cytoplasm enlargement assay. S-hCSC was significantly increased X-gal positive cells ($p<0.01$) and their cytoplasm enlarged compared control groups ($p<0.01$). In addition, senescence associated protein p53 inhibitor molecule, Mdm2, was significantly reduced in S-hCSC rather than in control ($p<0.05$). Therefore, proliferation capability was significantly reduced in S-hCSC compared to control as shown ($p<0.01$). These results due to the cell cycles G1 arrest in S-hCPC; Cyclin E/CDK2 expression was reduced. To confirm the expression of MAPK during hCPC senescence, we analyzed by western blot. Interestingly, only ERK was strongly phosphorylated in S-hCPC rather than in control group ($p<0.01$). With this in mind, we examined whether hCSC senescence prevent by treatment with ERK inhibitor as U0126. X-gal positive S-hCSC significantly reduced after treatment with U0126 for 24h ($p<0.05$) and 48h ($p<0.05$). Therefore, irregular morphology of S-hCSC was recovered after treatment with U0126 for 24h and 48h. In addition, reduced in vitro amplification capability of S-hCSC was clearly recovered after treatment with U0126 for 24h ($p<0.01$) and 48h ($p<0.01$). The senescence associated protein p53 inhibitor, Mdm2 expression was significantly increased after treatment with U0126 for 24h and 48h which compared to S-hCPC ($p<0.01$). Finally, we confirmed hCSC differentiation potential; we induced transdifferentiation into endothelial, smooth muscle cell, and cardiomyocyte *in vitro*. However, there was any difference protein expression among control, S-hCSC, and treatment with U0126. Based on these results, we concluded that hCSC senescence processed via MAPK and p53 mediated signaling pathway, and senescence hCSC prevented using by MAPK regulation especially, ERK inhibition. Therefore, this study may offer strong evidence for basic research of human cardiac stem cell senescence and clinical application for stem cell therapy of cardiovascular disease.

Poster Board Number: T-2010

CHARGED SURFACE MODIFICATION CULTURE SYSTEM FACILITATES MAINTENANCE OF HUMAN C-KIT POSITIVE CARDIAC STEM CELLS

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Ischemic heart diseases accelerate damage and loss of cardiomyocyte. Replacing *de novo* human cardiomyocyte is difficult; because of human adult cardiomyocyte has limited regenerative capability. However, accumulating evidences suggested that damaged cardiomyocyte could replace using by transplantation of some adult stem cells. Recently, cardiac stem cells (CSC), one potential source of stem cells, were identified. These stem cells have multipotent capability which could be transdifferentiated into cardiomyocyte, endothelial lineage and smooth muscle cell lineages. However, in fact, CSC has a low expansion ratio and is hardly difficult to maintain in canonical stem cell culture vessels. Therefore, we were designed to examine the efficacy of a novel CSC culture method that uses surface-charged modification of culture vessels which coated by charging molecules such as carboxyl and amine residue. Thus, the aim of this study was we examined effects of in vitro amplification and attachment on isolated human c-kit positive cardiac stem cell (hCSCc-kit+). In this study, we used the hCSCc-kit+ were isolated from human infant myocardium using a c-kit antibody. The experimental groups divided into following three groups: hCSCc-kit+ cultured on canonical non-coated culture vessels (control), carboxyl residue-coated vessels (carboxyl), and amine residue-coated vessels (amine). To confirm the effects of surface-charged modification culture vessels on hCSCc-kit+, we first examined cellular proliferation. The proliferation ratio in amine group was significantly increased rather control ($p<0.05$), and carboxyl group as shown ($p<0.05$). Next, we examined the c-kit expression maintenance during culture using by fluorescence-activated cell sorting. The maintenance of c-kit expression capability in amine group greater extended rather than control as shown ($p<0.01$). In addition, carboxyl group also extended c-kit expression maintenance rather than control group ($p<0.05$). These results suggested that hCSCc-kit+ cultured on amine and carboxyl residue-enriched culture vessels were facilitates maintenance of stem cell potency compared to other culture vessels as non-coated and carboxyl. Furthermore, we observed that cellular attachment capability in amine group greater than control ($p<0.05$). Finally, we confirmed cellular signaling molecules expressions. The amine and carboxyl groups increased phosphorylation signals, such as focal adhesion

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kinase (FAK) and cytosolic sarcoma (c-Src), and enhanced ERK/CDK2 signaling. There was a significantly down-regulation of the stress signal transducer, JNK, in amine group. These results suggested that hCSCc-kit+ cultured on charged surface modification increased FAK and c-Src phosphorylation, and enhance cellular proliferation and cellular attachment. Enhanced cellular attachment increased cellular survival pathway and reduced cellular stress response signaling pathway. On the basis of our results, we conclude that amine residue-enriched surface modification may improve hCSCc-kit+ in vitro amplification. This surface modification culture vessels improved cellular proliferation and attachment during in vitro hCSCc-kit+ culture, possibly through modulating intracellular signal transducers. Therefore, this novel culture methodology may be clinically applicable to cell therapy based on stem cells for heart disease.

Poster Board Number: T-2011

AN IMPORTANT ROLE FOR A MITOCHONDRIAL REGULATOR IN DETERMINING HESC-DERIVED CARDIOMYOCYTE IDENTITY

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A fundamental feature of cardiomyocyte development in the body is the large increase in mitochondrial content from the fetal to the mature cell. These organelles are ultimately required to fund the high ATP demand of cardiac work. It is not known for certain which genes co-ordinate the increase in mitochondria or how important this programme is for determining the overall cell phenotype, in addition to increasing ATP-generating capacity. In this study, we addressed the importance of this process in human embryonic stem cell (hESC)-derived cardiomyocytes, with the long term aim of elucidating the conditions required to mimic *in vivo* development conducive with cell maturation. Using an NKX2.5-GFP hESC reporter line allowing identification and sorting of cardiomyocytes, we show that increasing mitochondrial biogenesis is indeed a fundamental and specific feature of these developing cells. The gene PGC-1alpha, an important regulator of mitochondrial biogenesis, is specifically upregulated in cardiomyocytes and its knockdown by RNAi in these cells blocks the increase in mitochondrial density. In tandem with mitochondrial changes we discovered that cardiac gene expression is repressed early on PGC-1alpha knockdown within NKX2.5 positive cells, suggesting a close connection between the function of this protein and cardiomyocyte identity. We find that the long-term consequences of the genetic manipulation depend on the subsequent level of cell stimulation and vary between a mild and extreme disturbance in the cell phenotype. These results indicate a novel finding - that the regulation of mitochondrial function and cardiac gene expression are connected. Whether this is determined directly by the regulator or indirectly via mitochondrial functionality is being explored. This finding has important consequences for any downstream applications involving these cells, especially in the study of stress-related diseases such as cardiac hypertrophy or mitochondrial disease.

Poster Board Number: T-2012

A NOVEL METHOD OF SELECTING HUMAN EMBRYONIC STEM CELL-DERIVED CARDIOMYOCYTE CLUSTERS FOR ASSESSMENT OF POTENTIAL TO INFULENCE QT INTERVAL

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Application of human embryonic stem (ES) cells and induced pluripotent stem (iPS) cells is opening the new era of drug discovery and toxicological testing. The most promising cell type is cardiomyocytes differentiated from these stem cells, because it is difficult to get human primary cardiomyocytes. QT prolongation, which is a risk factor of ventricular tachycardia, is a critical cause of the withdrawal or use restriction of marketed drugs. We have used hERG channel-overexpressing cells as an *in vitro* model and dogs or monkeys as *in vivo* models to predict QT prolongation. However, there are discrepancies between the results of *in vitro* hERG assays and *in vivo* QT experiments. Thus, we consider that desirable evaluation systems are those that utilize human cardiomyocytes physiologically expressing multiple ion channels involved in QT intervals. In our preliminary experiments of QT risk evaluation using a multielectrode recording system, we found that responsiveness of field potential duration (FPD) to reference compounds differed very much from non-responders to excessive responders in human ES cell-derived cardiomyocyte clusters. We report a novel method selecting clusters suitable to the evaluation of drug candidate compounds to establish a robust QT risk assessment system. Clusters were treated with cisapride, a hERG channel blocker, at 100 nM, and selected with criteria of 5 to 20% of corrected FPD (FPDc) prolongation. After washout for several days, selected clusters were treated with reference compounds. FPDc was prolonged by blockade of hERG channel (+12.1% by E-4031 at 30 nM; +6.0% by *dl*-sotalol at 10 μM) and KvLQT1 channel (+9.2% by chromanol 293B at 10 μM; +10.0% by HMR1556 at 1 μM), and by activation of sodium channel (+15.5% by veratridine at 1 μM) and calcium channel (+13.9% by Bay K8644 at 30 nM). FPDc was shortened by blockage of calcium channel (-13.4% by verapamil at 30 nM; -10.8% by nifidipine; -10.3% by diltiazem) and by activation of K_{KATP} channel (-5.5% by pinacidil at 1 μM). Quinidine and dysopyramide prolonged FPDc by 8.2% at 1 μM and 9.8% at 10 μM, respectively. Selected clusters are proper to assess effects of compounds on ion channels affecting QT intervals. This is the first report on the establishment of the QT risk assessment system using pharmacologically selected clusters. In addition, our results show again usefulness of stem cell technologies.

Poster Board Number: T-2013

HUMAN CARDIAC PERICYTES: MULTIPOTENT MESODERMAL PROGENITORS WITHIN THE MYOCARDIUM EXHIBITING TISSUE SPECIFICITY IN MYOGENESIS

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Microvascular pericytes purified from multiple human tissues have recently been shown to harbor stem cells exhibiting myo-, osteo-, chondro- and adipogenic differentiation potentials and therefore

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suggested to be one of the developmental origins of mesenchymal stem/stromal cells (MSCs). Nevertheless, it is not clear whether microvascular pericytes residing in the human heart possess multipotency and stem/progenitor cell characteristics, similar to their developmentally distinct counterparts in other organs. Herein we explored the hypothesis that pericytes are present within the human myocardium as multi-lineage mesodermal progenitors with tissue specificity. Immunohistochemistry showed that human adult and fetal cardiac microvascular pericytes express known pericyte markers *in situ*, including CD146, NG2, PDGFR-β, SMA, and SM-MHC, but not CD117, CD133, and endothelial cell markers. Human heart cells were isolated from ventricular myocardial biopsies by mechanical dissociation and collagenase digestion and subsequently subjected to fluorescence-activated cell sorting (FACS) for purification. Using a combination of selective cell surface markers, including positive selection marker CD146 and negative selection markers: CD34, CD45, CD56, and CD117, we prospectively purified human cardiac pericytes to homogeny. Sorted cardiac pericytes ($CD146^+CD34^-CD45^-CD56^-CD117^-$) can be cultured in the long term and displayed morphological and proliferative profiles similar to pericytes purified from skeletal muscle. Cultured cardiac pericytes consistently expressed CD146, NG2, PDGFR- and -β, SMA, and alkaline phosphatase and formed capillary-like networks in Matrigel at different passages. Cellular interaction between cardiac pericytes and endothelial cells was further illustrated by the formation of capillary networks in two- and 3-dimensional Matrigel co-cultures. To examine their cardiomyocyte differentiation capacity, cultured cardiac pericytes were treated with 10 M 5-Azacytidine (AZA) for 72 hours and subsequently maintained in differentiation medium for up to 2 weeks. Immunocytochemistry showed that a fraction of AZA-treated cardiac pericytes exhibit -sacromeric actinin, cardiac myosin heavy chain, and nuclear GATA-4, but not cardiac troponin-I, suggesting an immature phenotype. Immunohistochemistry and flow cytometry showed that cardiac pericytes natively express classic MSC markers, including CD44, CD73, CD90, and CD105. To investigate whether these cells possess multi-lineage mesodermal potential, cardiac pericytes were cultured in osteogenic, chondrogenic, adipogenic, and skeletal myogenic conditions. Cardiac pericytes exhibited robust osteo-, chondro-, and adipogenesis, but not skeletal myogenesis. No myotube formation or expression of fast skeletal myosin heavy chain was observed, suggesting tissue specificity of cardiac pericytes, distinct from pericytes of other origins, in myogenesis. Our results showed that human cardiac pericytes can be prospectively purified by FACS with a combination of selective cell lineage markers and possess mesodermal multipotency except skeletal myogenesis. Currently we are investigating the differential activation of myogenic genes in cardiac pericytes after induction as well as the engraftment and cardiomyocyte differentiation of transplanted GFP-labeled cardiac pericytes within the healthy and ischemic myocardium in immunodeficient mouse models.

Poster Board Number: T-2014

DIRECTED FORMATION OF BIOENGINEERED HUMAN MYOCARDIUM (BHM) FROM PLURIPOTENT STEM CELLS

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Bioengineered human myocardium (BHM) has a vast range of current and potential applications including human developmental biology, pharmacological studies, disease modelling and regenerative medicine. Ordinarily, human tissue engineered myocardium is created by firstly differentiating pluripotent stem cells into cardiomyocytes and also deriving/isolating other required cell types (eg fibroblasts), and subsequently forming tissue. In this investigation we hypothesised that we could form the BHMs directly with undifferentiated pluripotent stem cells and instructing the formation of myocardial muscle including all the required cell types.
Methods and Results: We firstly developed a consistent differentiation protocol capable of producing large numbers of cardiomyocytes and stromal support cells/fibroblasts simultaneously. This was achieved using a staged and chemically defined protocol in 2D (16 d). The resulting differentiation cultures (n = independent experiments) were then characterised using flow cytometry demonstrating high yields of cardiomyocytes (α-actinin positive cells, 28 ± 4 % SEM, n = 10), high yields of stromal cells characterised by α-smooth muscle actin (82 ± 6 % SEM) and collagen I (53 ± 5 % SEM) and low yields of remaining pluripotent stem cells (TRA-1-60/OCT4 positive cells, 0.1 ± 0.05 % SEM). In addition, there were low levels of potentially contaminating cell types using qPCR (n = 3) for primitive endoderm (SOX17), pluripotent stem cells (OCT4), primitive mesodermal cells (MESP1) and neural cells (NEUROD1). We then tested our hypothesis by applying the protocol to undifferentiated pluripotent stem cells in a collagen type 1 hydrogel, with the addition of mechanical loading to improve tissue maturity. Our hypothesis was confirmed and at the endpoint (23 d) the BHMs had a contractile force of 65 ± 15 SEM (n = 7, from 3 exp). The BHMs contained elongated cross-striated cardiomyocytes together with stromal cells as demonstrated in whole mount immuno-staining analysis. In addition, qPCR analysis revealed that the development of BHMs followed the temporal progression of known developmental pathways, with very low levels of other contaminating cell types. This analysis also revealed the early and transient expression of markers for heart progenitor populations, including the primary heart field (TBX5), secondary heart field (ISL1) and the pro-epicardial organ (GATA5). Flow cytometric analysis demonstrated that these constructs were comprised of mainly cardiomyocytes (α-actinin positive cells, 46 ± 5 % SEM, n = 9 from 3 exp) and stromal cells (30 ± 6 % SEM, α-smooth muscle actin positive cells and 4 ± 1 % SEM collagen I positive cells, n = 9 from 3 exp), with low yields of remaining pluripotent stem cells (TRA-1-60/OCT4 positive cells, 0.03 ± 0.01 % SEM, n = 4 from 3 exp). Conclusion: BHMs can be formed directly with undifferentiated pluripotent stem cells under "bio-instructing" conditions.

Poster Board Number: T-2015

PREDICTIVE TOXICOLOGY USING SPONTANEOUSLY CONTRACTING HUMAN IPSC DERIVED CARDIOMYOCYTES

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A large percentage of new drugs fail in clinical studies due to cardiac toxicity. Therefore development of highly predicative *in vitro* assays suitable for high throughput screening (HTS) is extremely important for drug development. Human cardiomyocytes derived from stem cell sources can greatly accelerate the development of new chemical entities and improve drug safety by offering more clinically relevant cell-based models than those presently available. Induced pluripotent stem cell (iPSC) derived cardiomyocytes are attractive because they express ion channels and demonstrate spontaneous mechanical and electrical activity similar to native cardiac cells. They are also available in quantities required for drug

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discovery and screening campaigns. We have developed a HTS compatible assay for testing toxicity and cardioprotective effects of drug candidates and developing drugs. Spontaneous contractions of iPSC derived cardiomyocytes (iCell Cardiomyocytes) in 96 or 384 multiwell plates are measured using a Ca2+ sensitive fluorescent dye. Rapid changes in fluorescence intensity associated with each beat event, or "sparks", are recorded using a fast kinetic whole plate imaging system (FLIPR Tetra System). Various parameters for each well of cardiomyocytes are automatically measured including beat frequency, average rise and decay times, average beat temporal duration, and irregularities in beat patterns. We have characterized a number of known compounds including b-adrenergic receptor agonists and antagonists, and ion channel blockers. We have used the method to determined IC50/EC50s of chronotropes (epinephrine, isoproterenol, propranonol, verapamil) and cardiotoxic compounds (doxazosin, imatinib, antimycin A, terfenadine, cisapride, astemizole) and found good agreement with previously reported values. The combination of a robust cell model and fast simultaneous whole-plate measurement offers excellent assay precision and can be a valuable tool to predict cardio toxic effects earlier in the drug development process.

Poster Board Number: T-2016

ARRHYTHMIA MODEL IN HUMAN ES/IPS CELL DERIVED CARDIAC CELL MONOLAYER

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Background: In order to advance stem cell research to the medical applications, it is crucially important to have as full knowledge of the differentiated tissue as possible. In our work, we study monolayers of human ES/iPS cell-derived cardiac tissue as a model system for prediction of arrhythmogenesis. Methods: Cardiomyocytes were differentiated from human ES cell (KhES3) and iPS cell (253G1) for 3-4 weeks, dissociated by proteases and seeded as cell layers (12 mm-diameter). They were confirmed as electrical syncytium using optical mapping with Ca2+ sensitive dye (Fluo-4). Quantitative real time polymerase chain reaction (PCR) and immunostaining were used to analyze these monolayers. We measured relative gene expression levels compared to GAPDH at 2, 7, and 28 days after seeding. Results: Excitation wave propagation was detected in the cell monolayer from 2 to 28 days after seeding. Propagation wave velocity was dependent on the stimulation cycle length and lidocaine concentration. The mean wave velocity was 67.4± 9.7, 44.0 ± 7.4, and 27.6 ± 2.7 mm/s when the lidocaine concentration was 0, 0.1, and 0.2 mM, respectively (n=7, 7 days after seeding, One-way ANOVA P=0.007). Reentrant (spiral) wave propagation was detected in the cell layers while applying high-frequency stimulation (n=25/77). The size of unexcitable spiral core increased depending on the concentration of E-4031 and nifekalant. There was significant difference of mean α-actinin positive area between the monolayer in which spiral wave was detected and the monolayer in which it could not be detected or disappeared easily (n=4 for each, 64.6% vs 83.3%, t test P=0.01). With quantitative PCR, the expression of β-MHC, Cx43, and HERG-1b increased, but that of α-MHC and HCN4 decreased during the time course. Conclusions: We succeeded to create powerful tool to evaluate the arrhythmogenic potential of cardiac tissue derived from human ES/iPS cells. This human ES/iPS cell-derived cardiac tissue is useful for *in vitro* reentrant arrhythmia model and drug screening.

Poster Board Number: T-2017

ULTRA-RAPID METHODS TO DETERMINE TRANSGENE PLACEMENT AND CARDIAC POTENTIAL OF NEWLY DERIVED HUMAN INDUCED PLURIPOTENT STEM CELLS

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Most reprogramming methods to generate human induced pluripotent stem cells (hiPSC) produce a large number of clones of which usually 2-3 are selected for further research. There are currently no rapid methods available to select particular clones with a limited number of inserts, integrated in genomic locations not critical to stem cell maintenance or differentiation. In addition, the clones of interest for further research will be those able to differentiate efficiently into the lineage of choice. Furthermore significant line-to-line variability in differentiation capacity is observed among both human embryonic stem cells (hESC) and hiPSC lines which can have important consequences for the progress of research. To address these issues, we developed a novel combination of methods to make informed choices on which hiPSC clones to select for studies on genetic cardiac disease, based on lentiviral vector insert number and location, and cardiac differentiation potential, all of which were determined within 3 weeks after initial picking of putative clones. As an alternative to Southern blotting, we used the splinkerette PCR technique in combination with sequencing to determine insert number and location of integrated reprogramming genes. Several lentivirally reprogrammed hiPSC clones (0.1-5 MOI) were tested and the outcomes were consistent with Southern blot results. The cardiac differentiation potential was determined using a new, fully defined monolayer differentiation protocol designed for use with early hiPSC colonies grown in mTeSR. This monolayer differentiation method resulted in beating cardiomyocytes within 12 days. No optimization was required when tested on numerous clones of 8 independent hiPSC and hESC lines. Cardiac differentiation efficiencies within clones ranged from 3 to 50%, as determined by intracellular FACS staining. This allowed cardiac potential to be determined on individual clones along with insert number and location information without the need to passage or scale up culture. In summary, we describe a fast and efficient method requiring minimal amounts of starting material for the identification of high quality hiPSC clones, based on number and location of integrated reprogramming genes and cardiac differentiation potential.

Poster Board Number: T-2018

DIFFERENTIATION OF CARDIOMYOCYTE PHENOTYPE FROM HUMAN DENTAL PULP STEM CELLS

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In recent years much effort is spent in attempts to cure cardiac diseases or improve such patients' condition. A new branch of medicine, called regenerative medicine, focuses on embryonic or adult stem cells to repair damaged cardiac vascular tissue. However,

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embryonic stem cell research still involves ethical issue and the usual source for adult stem cells, bone marrow stem cells are generally difficult to isolate and expand in culture. As a possible alternative to using bone marrow stem cells, we investigated the use of dental pulp stem cells (DPSC) extracted from human permanent teeth. Tooth surface were cut around the cementoenamel junction and then cracked open to reveal the pulp chamber. The pulp tissue was separated from the crown and root and then digested in 3mg/ml collagenase type I. DPSC were seeded in flasks, magnetically separated and only CD117 positive cells were used in the recent study. Cells were grown as monolayer in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% FBS (fetal bovine serum) at 37°C and 5% CO2. Every four passages the CD117 positive fraction was separated magnetically. In order to differentiate DPSC into cells with characteristics of cardiomyocytes, we treated DPSC with the demethylating agent 5-azacytidine and two differentiation growth media - HM1 and HM1 (HM1: DMEM, 2%FBS, 20nM dexamethasone, 100µM L-ascorbic acid, 10ng/ml LIF (leukemia inhibitory factor); HM2: DMEM, 2%FBS, 20nM dexamethasone, 100µM L-ascorbic acid, 25ng/ml BMP-2 (bone morphogenetic protein 2), 50ng/ml bFGF (basic fibroblast growth factor), 2ng/ml IGF-I (insulin-like growth factor 1)). Flow cytometry test showed that concentrations up to 10µM 5-azacytidine cannot induce significant levels of apoptisis in DPSC. Therefore, part of the DPSC were exposed to 5µM 5-azacytidine for 24 hours one day after seeding, prior changing the media with HM1. After that all cells were grown in HM1 for three weeks followed by HM2 for another one to three weeks. After four to six weeks, cells changed their morphology and showed several characteristics typical for cardiomyocytes. Immunocytochemical tests were positive for GATA-4 (GATA binding protein 4), Nkx-2.5 (Nk2 transcription factor related locus 5), DES (desmin) and cTnT (cardiac Troponin T). Analysis with real time RT-PCR showed significant increase in the expression of BMP-2, BMP-4 (bone morphogenetic proteins 2 and 4), BMPR1A (bone morphogenetic protein receptor type 1A), MEF2C (myocyte enhancer factor 2C), MYH7B (myosin heavy chain 7B), as well as SMAD1 and SMAD5 (SMAD family members 1 and 5). All those transcription factors are associated with mature cardiomyocytes. Analysis of specific markers at the protein and mRNA levels demonstrated that cardiomyogenesis can be induced in dental pulp stem cells in certain growth conditions. Our novel approach of using teeth makes it possible to produce enough number of the cells for future transplantation studies. We are confident in its value for patients with cardiac problems.

Poster Board Number: T-2019

DEFINING CELL LINEAGE RELATIONSHIPS DURING HUMAN CARDIOGENESIS

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NKX2-5 is expressed in the heart throughout life. We targeted sequences encoding green fluorescent protein (GFP) to the NKX2-5 locus of human embryonic stem cells (hESCs). *NKX2-5*$^{GFP/w}$ hESCs facilitate quantification of cardiac differentiation and purification of hESC-derived committed cardiac progenitor cells (hESC-CPCs) and cardiomyocytes (hESC-CMs). Gene expression studies demonstrated that NKX2-5$^+$ hESC-CPCs and CMs constitute developmentally distinct populations. Furthermore, clonal analysis showed that NKX2-5$^+$ CPCs are capable of giving rise to the three major lineages in the heart, namely cardiomyocytes, smooth muscle and endothelium. GFP$^+$ CMs display an foetal-like action potentials, correlating with the gene expression profile. We have used NKX2-5$^+$ cells to identify VCAM1 and SIRPA as novel cell surface markers expressed on cardiac lineages. Flow cytometric temporal profiling of these three markers suggests a progression from a multipotent SIRPA$^+$ population to a myogenically committed NKX2-5$^+$SIRPA$^+$VCAM$^+$ population. Triple positive cells are contractile and express markers of CMs, whereas NKX2-5$^+$SIRPA$^+$ cells also express endothelial and smooth muscle markers. In addition, cultured NKX2-5$^+$SIRPA$^+$ cells give rise to NKX2-5$^+$SIRPA$^+$VCAM1$^+$ CMs. Furthermore, we have identified an NKX2-5$^+$CD34$^+$ cell population, which, when cultured, gives rise to endothelial cells. Therefore, these markers represent tools to investigate the molecular control of lineage specification during human cardiogenesis. *NKX2-5* is likely to play a key role in the differentiation of hESC derived cardiac cells. In order to examine *NKX2-5* function we generated hESCs in which both *NKX2-5* alleles have been disrupted. Future studies will utilise our *NKX2-5* allelic series to focus on the role of *NKX2-5* in regulating cell lineage specification and differentiation in human heart development.

Poster Board Number: T-2020

HUMAN INDUCED PLURIPOTENT STEM CELL -BASED MODEL FOR CATECHOLAMINERGIC POLYMORPHIC VENTRICULAR TACHYCARDIA

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Induced pluripotent stem cells (iPSC) can be generated by reprogramming differentiated cells into a pluripotent state, providing a way to study the pathophysiology of various genetic diseases. Catecholaminergic polymorphic ventricular tachycardia (CPVT) is an inherited cardiac disorder characterized by stress-induced ventricular tachycardia and risk of sudden death with a structurally normal heart. Here we introduce a functional cell model for CPVT caused by a mutation in the cardiac ryanodine receptor (RyR2) gene. This mutation interferes with RyR2 function, leading to diastolic leak of calcium from the sarcoplasmic reticulum (SR). We investigated whether the electrophysiological characteristics of this mutation can be mimicked *in vitro* by using iPSC-derived human cardiomyocytes. Spontaneously beating cardiomyocytes were differentiated from iPSC lines derived from a CPVT patient carrying the P2328S mutation in RyR2 and from one healthy control donor. Calcium handling and electrophysiological properties were studied by comparing mutation-specific and control cardiomyocytes by calcium imaging and patch-clamp techniques. We found substantial defects in electrical activity and calcium signaling in CPVT cardiomyocytes, presumably reflecting the cardiac phenotype observed in the patients. Catecholaminergic stress in CPVT cardiomyocytes led to various types of abnormal calcium transients and arrhythmias. CPVT cardiomyocytes displayed also a reduced SR calcium content, implicating leakage of calcium from the SR. Our cell model displayed aberrant calcium signaling consistent with well-known CPVT characteristics as well as abnormal electrophysiological properties that have not been reported before. Thus, this cell model for CPVT provides a promising platform to study basic

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pathology, to screen drugs safely, and to optimize drug therapy in a patient-specific manner.

Poster Board Number: T-2021

DIRECT CONVERSION OF HUMAN DERMAL FIBROBLASTS INTO CARDIOMYOCYTES USING RECOMBINANT TRANSDUCIBLE PROTEINS

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Although the transformation of differentiated cells to induced pluripotent stem cells (iPSCs), is a powerful approach, it has several limitations, including the low efficiency of the process and the necessity of forced expression of at least one pluripotent stem cell transcription factor. These methods involve the genomic integration of the transcription factor(s)' genes and thus harbor the risk of mutagenesis and tumorogenicity. A recent report showed the successful and efficient generation of cardiomyocytes from murine embryonic fibroblasts by brief reactivation of reprogramming factors. This allows fibroblasts to enter an unstable intermediate "partial reprogramming" phase, after which the cells were directed toward cardiomyocytes. Although this protocol offers fast conversion of fibroblasts to cardiomyocytes, there are still many risks associated with genomic integration of transcription factors' genes. In addition, the direct conversion is only carried out on murine fibroblasts and the protocol`s effectiveness on human dermal fibroblasts is not evaluated yet. In the present study we treated human dermal fibroblasts (HDFs) with a combination of 6 proteins (including Oct4, Klf4, Sox2, and c-Myc) conjugated with HIV TAT for 12 days in order to enter HDFs into a partially reprogrammed state. During the first 8 days, cells were treated with reprogramming media containing protein mixture and 15% FBS and 5% knockout serum replacement (KO-SR), followed by a switch to 1% FBS and 14% KO-SR for 4 days. After this period, cells were treated with BMP4 (20 ng/ml) in a serum-free medium for additional 4 days followed by 3 days of Chir99021 (a GSK3 inhibitor) treatment. After this period cells were collected for real-time PCR analysis. Cells treated with recombinant proteins showed significantly higher expression of early-stage and late-stage cardiac markers such as GATA-4, Mef2C, NKX2.5, alpha Myosin Heavy Chain (α-MHC), Cardiac Troponin T (cTnT) and alpha cardiac actin, in comparison to HDFs that were only exposed to BMP4 growth factor. Results were confirmed with immunocytochemistry for GATA-4, NKX2.5, cTnT and α-MHC. CONCLUSION. We have successfully generated cardiomyocytes from Human Dermal fibroblasts using recombinant cell-penetrating reprogramming proteins. This technique offers safe and fast generation of cardiomyocytes for future applications in regenerative medicine as well as drug screening. Furthermore this novel tool might be a potential method for *in vivo* conversion of ischemia-triggered aggregation of fibroblasts into cardiomyocytes in infarcted heart.

Poster Board Number: T-2022

ENDOTHELIAL CELLS PROMOTE CARDIAC STEM CELL THERAPY USING MOUSE EMBRYONIC STEM CELL-DERIVED CARDIOMYOCYTES

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To avoid undesired cell types when isolating embryonic stem cell (ESC)-derived cardiomyocytes (ESC-CMs), we used mouse ESCs stably transfected with a cardio-specific alpha-myosin heavy chain-driven enhanced green fluorescence protein (EGFP) reporter. We further found that coculture with endothelial cells enhanced the maturation indexes of ESC-CMs, including cell size, axis alignment, sarcomere organization, contraction rate and the expression of the gap junction protein connexin-43, through direct cell-to-cell contact. Here we examined whether co-transplantation of ES-CMs with endothelial cells had therapeutic effects using a mouse model of myocardial infarction. We found that cotransplantation with endothelial cells, but not fibroblasts, improved ESC-CM cell engraftment following injection. Moreover, cotransplantation with endothelial cells significantly increased ESC-CM maturation, as indicated by increases in both cell alignment and size. We then used echocardiography to determine the left ventricular ejection fraction and the end-systolic and end-diastolic volumes of mice which underwent experimental myocardial infarction followed with injection of saline, peptide nanofibers (NFs, as a cell carrier), ESC-CMs alone or along with endothelial cells or fibroblasts. At day 21, in infarcted hearts receiving NFs containing ESC-CMs plus endothelial cells improved ejection fraction ($P < 0.05$), decreased left ventricular end-systolic and end-diastolic volumes ($P < 0.05$) and reduced the infarct size of hearts ($P < 0.05$). Together, these results suggest that co-transplantation of endothelial cells with ESC-CMs promotes the maturation of ESC-CMs *in vivo* and improves cardiac cell therapy after myocardial infarction. Hence, the co-transplantation of ESC-CMs with endothelial cells may pave the way for future success in using stem cell therapy for cardiac regeneration.

Poster Board Number: T-2023

ABLATION OF THE HCN4 ION CHANNEL IN DIFFERENTIATING HUMAN INDUCED PLURIPOTENT STEM CELL-DERIVED CARDIOMYOCYTES VIA ZINC FINGER NUCLEASE MUTATION

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Cardiovascular disease remains a leading cause of death worldwide each year. Stem cell-derived cardiomyocytes hold great promise in tissue engineering for cardiac regeneration. However, current protocols for the differentiation of pluripotent stem cells toward cardiomyocyte lineages result in heterogeneous populations of cardiac cell types, in which spontaneous contraction is observed. Even among cardiomyocytes, atrial, ventricular, and pacemaker phenotypes are presents. Spontaneously depolarizing stem-cell derived cardiomyocytes (SC-CMs) initiate contraction of surrounding cardiomyocytes. Control over electrical activity of differentiating SC-CMs, either by blocking spontaneous activity or by providing exogenous electrical stimulation, has been shown to reduce heterogeneity of resulting cell populations. Additionally, spontaneous pacemaker activity represents a potential risk for causing deadly arrhythmias if SC-CMs are to be used clinically. The hyperpolarization-activated cyclic nucleotide-gated 4 (HCN4) ion channel is known to

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be partially responsible for initiating contraction in cardiac nodal tissue. This pacemaking activity, if present in ventricular tissues, might lead to undesired arrhythmias. We hypothesize that ablating the HCN4 gene in human induced pluripotent SC-CMs (hiPSC-CMs) will result in production of homogenous ventricular-type cell populations lacking spontaneous contractility. To accomplish this, zinc finger nucleases (ZFNs) represent a powerful and clinically-relevant technology capable of specifically targeting and abolishing gene expression. In this project four plasmid vectors (two dimeric pairs) harboring ZFNs, designed specifically for targeting exon one of the HCN4 gene, were constructed using recombinant DNA techniques. Each ZFN was electroporated into (IMR90)-1 hiPSCs. Colonies were screened for biallelic mutation of HCN4, lack of HCN4 protein expression, and propensity to differentiate into cardiomyocytes. We postulate that HCN4-/- cardiomyocytes will display a ventricular gene expression profile and electrophysiological phenotype, resulting in cell populations better suited for use in drug testing and in tissue engineering clinical applications for studying and treating several diverse forms of heart disease.

Poster Board Number: T-2024

ISOLATION AND CHARACTERIZATION OF MOUSE CARDIAC STEM CELLS AS GREEN FLUORESCENT PROTEIN-RETAINING CELLS

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The heart contains a reservoir of stem and progenitor cells. Cardiac stem/progenitor cells have been characterized by the expression of cell surface markers such as Sca-1 and c-Kit or transcriptional activation of genes such as Isl-1 and Wt1. Alternatively, cardiac stem/progenitor cells can be defined as side population (SP) cells, which are characterized by their unique ability to efflux a DNA-binding dye, e.g. Hoechst 33342. Despite these marker proteins and genes as well as functional definition, the origin and identity of cardiac stem cells remain only poorly understood. In this study, we aimed to identify and characterize cardiac stem/progenitor cells as "label-retaining cells (LRCs)". Tissue stem cells proliferate infrequently and therefore the labels (typically BrdU), once incorporated into their DNA or nucleosomes, are retained for an extended period, while the labels incorporated into transit amplifying cells become quickly diluted as the cells continue to proliferate. Given the difficulty to isolate BrdU-retaining cells without losing their viability, we employed GFP to fluorescently label slow-cycling cells by expressing histone H2B fused-GFP under the control of a tetracycline-responsive regulatory element (TRE). The TRE-H2BGFP transgenic mice (kindly provided by Dr. Elaine Fuchs) were crossed with the mice expressing reverse tetracycline transactivator driven by the ROSA26 promoter, yielding double transgenic mice that express GFP in a wide variety of cell types only when the mice are given doxycycline. The double transgenic mice were given doxycycline through mother's drinking water for two weeks from E10.5 through P7 and chased for up to 12 weeks. GFP-labeled cells were detected ubiquitously in the heart just after the administration of doxycycline; however, the labeled cells were markedly decreased in number after six weeks of chase and were predominantly localized in the epicardium and near coronary vessels. To characterize the GFP-LRCs, the cells expressing GFP after six weeks of chase were sorted from whole cell suspension of the heart using a fluorescence-activated cell sorter. Cells expressing a high level of GFP comprised ~0.5% of whole dissociated cells. Quantitative RT-PCR analyses revealed that the GFP-retaining cells expressed high levels of epicardium-associated markers, i.e., podoplanin, Tbx18 and Wt1, indicating that a majority of GFP-retaining cells are derived from the epicardium. GFP-retaining cells also expressed a panel of cardiac stem/progenitor markers including Sca-1, c-Kit and GATA4, the expression levels of which were equal to or higher than those of SP cells isolated from the adult heart. These results, taken together, indicate that the GFP-retaining cells we isolated represent a major population of cardiac stem/progenitor cells residing in the epicardium and are therefore a promising source for functional characterization of cardiac stem/progenitor cells in myocardial development and regeneration.

Poster Board Number: T-2025

TOWARDS THE LABEL-FREE PURIFICATION OF STEM CELL-DERIVED CARDIOMYOCYTES USING SECOND HARMONIC GENERATION

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In the translation of regenerative cardiology from bench to bedside, the development of a label-free, sensitive method for analyzing, counting and sorting viable human pluripotent stem cell-derived cardiomyocytes (PSC-CMs) of varied maturity is an important step yet to be taken. In order to use PSC-CMs for transplantation-based regenerative therapies or for engineering cardiac tissue for *in vivo* use, one must be able to control the phenotype, number and purity of CMs used so that reproducible and clinically acceptable tissue properties are achieved. We have taken the first steps towards realizing a label-free PSC-CM sorting methodology by utilizing second harmonic generation (SHG), a nonlinear optical phenomenon that occurs in the rod domains of sarcomeric myosin. PSC-CMs have a strong capacity to generate the SH signal, while undifferentiated stem cells do not. SHG intensity, when integrated over entire cell volumes, is strongly dependent on the development of sarcomere architecture in PSC-CMs. The effect was observed in PSC-CMs from the same differentiation day that were re-plated for varying lengths of time, as well as in suspension PSC-CMs fixed at different differentiation time points. Additionally, a PSC-CM's capacity for SHG is retained even after single cells have been retained in suspension for two hours. The potential specificity of using SHG to identify PSC-CMs is demonstrated to be > 97%. Furthermore, it is shown that other cells that arise in a typical cardiac-directed stem cell differentiation protocol, including smooth muscle cells, do not generate SH signals. Based on our observations thus far, we have started to develop a flow cytometer based on SHG by integrating an appropriate optical scheme with microfluidic devices in order to obtain enriched, pure populations of PSC-CMs in a label-free, non-genetic manner.

Poster Board Number: T-2026

A NOVEL CARDIAC *EX VIVO* CULTURE SYSTEM REVEALED THE ELONGATION OF CELL CYCLE PHASES IN CARDIOMYOCYTES DURING DEVELOPMENT

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Background: Although cardiomyocyte cell cycle regulatory mechanisms in molecular medicine have been studied, little is known

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about the spatial and temporal patterns of cardiomyocyte cell cycle progression in development. One major reason is that prior methods in cell cycle analysis mostly need sample fixation. By interlocking fluorescent probes with antiphase oscillating proteins that mark cell-cycle transitions, novel technique "Fucci" (fluorescent ubiquitination-based cell cycle indicator) effectively labeled individual G1 phase nuclei red, S/G2/M phase green and G1 /S transition phase yellow in living cells. Therefore Fucci is an useful tool to evaluate native cardiomyocyte cell-cycle, but to our knowledge, there are no standard protocols of live imaging in *ex vivo* heart model. The purpose of our study was to establish an cardiac *ex vivo* culture system in order to investigate the native cell-cycle dynamics in cardiomyocytes by applying Fucci. Methods and Results: We first confirmed that Fucci probes effectively highlight the cell cycle in cardiomyocytes. Cardiomyocytes in Fucci-expressing embryos at E18.5 (E0 = the day of plug) were immunostained for PCNA and PHH3, which are known as classical markers of S and M phase respectively. Both PCNA and phosphohistoneH3 positive cardiomyoctes were also Fucci green positive. On the other hand, Fucci red positive cardiomyocytes did not show signals of PCNA or phosphohistoneH3. Compared with classical methods, these results support that Fucci probes effectively highlight the cell cycle phases in cardiomyocytes. Next, we examined cardiomyocytes of Fucci-expressing transgenic mice during development. Fucci probes indicated that the S/G2/M phase cardiomyocyte population decrease during development (19.0% ± 5.3% at E11.5, 2.5% ± 1.0% at post natal day1). We also report that the compact layer has a higher population of S/G2/M phase cardiomyocytes than the trabecular layer in early development (E11.5 compact layer; 56.1% ± 9.9%, trabecular layer; 20.5% ± 3.3%). These consistent findings also strongly support Fucci as a novel method to analyze cardiomyocyte cell cycle. For establishing an *ex vivo* culture system, embryos and neonates were anesthetized by hypothermia, and the heart was transferred to liquefied low melting agarose gel immediately. On an ice bedded condition, cardiac slices (200μm-300μm) were made horizontally, and cell cycle transition was examined by 5-ethynyl-20-deoxyuridine (EdU) staining for 24 hours. The numbers of Edu positive cardiomyocytes showed that our method successfully replicated the in vivo cell cycle transition in our *ex vivo* culture system (*ex vivo* 27.4% ± 5.4%, in vivo 29.1% ± 8.9%). Finally we analyzed cardiomyocyte cell cycle dynamics applying the *ex vivo* culture method to Fucci-expressing transgenic mice. Live imaging visualized the cell cycle progression of cardiomyocytes and we assessed the length of S/G2/M phase in cardiomyocytes. Interestingly, S/G2/M phase length in cardiomyoctyes elongated during development (E14.5; 11.4 h ± 1.3 h, post natal day 1; 14.8 h ±1.5h). Thus, we succeeded in establishing an cardiac *ex vivo* culture system and by applying new technique Fucci, we elucidated the native cell-cycle dynamics in cardiomyocytes. The elongation of S/G2/M phase in cardiomyocytes could be a sign of maturation during development, and further analysis will lead us to a new approach for investigation of cardiac stem cells.

Poster Board Number: T-2027

NOVEL CHEMICALS POTENTLY INDUCING CARDIOMYOCYTES FROM VARIOUS PROGENITOR POPULATIONS

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Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are promising cell sources for cardiac regenerative medicine. Previously, we established a 2-dimentional culture-based sequential cardiovascular differentiation system from mouse ESC/iPSCs. This method is amenable to assess differentiation efficiencies at each differentiation stage (undifferentiated ESC/iPSCs, mesoderms, cardiac progenitors, cardiomyocytes). Recently, chemical biological approaches are starting to have an increasingly important role in stem cell biology and regenerative medicine. Some small molecules can efficiently regulate cell fate or modulate cell reprogramming. We reported that an immunosuppressant, cyclosporin-A, showed a novel potent cardiogenic effect specifically acting on Flk-1 positive mesoderm cells to increase cardiomyocytes by 10 times. We recently established a high-throughput screening (HTS) based on our ESC cardiovascular differentiation system and chemical approach. Using this HTS of co-culturing mouse ES cells that carry α-myosin heavy chain promoter-driven EGFP gene with OP9 mouse bone marrow-derived stroma cells, we can accurately and efficiently identify chemicals promoting cardiomyocyte differentiation from Flk-1 positive mesoderm. Here we report that we successfully discovered several cardiomyocyte differentiation chemicals (CDCs) from chemical libraries. In a natural chemical library derived from marine invertebrates, we identified a couple of natural chemicals (nCDC). Particularly, nCDC1 showed potent cardiomyocyte induction at nanogram/milliliter (ng/ml) level. This active concentration (2 ng/ml) was 1000 times lower than cyclosporin-A. And nCDC1 increased cardiomyocyte percentage and cell number that appeared from Flk-1 positive mesoderm cells approximately 20 times more than control. Next, we examined direct effect of nCDC1 on Flk-1 positive mesodermal cells and ES cell-derived cardiac progenitor cells, $Flk1^+/CXCR4^+/VE^-$cadherin– (FCV) cells. nCDC1 potently induced cardiomyocytes from $Flk\text{-}1^+$ cells and FCV cells even in the absence with OP9 stromal cells. We further examined effects of nCDC1 on a somatic cardiac progenitor population, cardiac side population (CSP) cells. nCDC1 induced differentiation of rat neonate CSP cells into cariomyocytes. These results showed nCDC1 induced cardiomyogenesis from mesodermal cells, cardiac progenitor cells derived from both ES/iPS cells and cardiac tissue. These findings would provide a clue for cardiomyocyte differentiation mechanisms and offer novel cardiac regenerative strategies.

Poster Board Number: T-2028

GENERATION OF CARDIOMYOCYTE-LIKE CELLS FROM NONMYOCYTES BY DEFINED FACTORS *IN VITRO* AND VIVO

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Cardiomyocytes are terminally differentiated cells and adult hearts have very limited regenerative capacity. Regenerative medicine using iPS cell-derived cardiomyocytes is an attractive therapy but still refinement is needed before clinical application. We recently found that mouse cardiac fibroblasts can be directly converted into cardiomyocyte-like cells by introduction of three cardiac-specific transcription factors, Gata4, Mef2c, and Tbx5 (GMT). We have now investigated whether direct injection of cardiac reprogramming factors into mouse hearts after myocardial infarction can convert endogenous cardiac fibroblasts into cardiomyocytes. Retroviral vectors containing cardiac reprogramming factors were used for gene therapy after coronary artery ligation. We found that GMT treatment induced cardiac gene upregulation and cardiac marker expression in fibroblasts. However, the induced cardiomyocytes by GMT did not have clear sarcomeric structures. Next, we screened additional cardiac reprogramming factors in human fibroblasts

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and found that addition of two factors to GMT enhanced cardiac generation and maturation. We next used 5 factors (GMT plus two factors) for *in vivo* reprogramming and found that the induced cardiomyocytes got mature and exhibited clear sarcomeric structures. These results indicate that endogenous cardiac fibroblasts can be a cell source for new cardiomyocytes by local delivery of defined factors, but modification of reprogramming factors may be needed to enhance cardiomyocyte generation.

Poster Board Number: T-2029

A CERTAIN MICRORNA SPECIES INDUCE CELL CYCLE PROGRESSION AND MITOTIC DIVISION IN NEONATAL AND ADULT RAT CARDIOMYOCYTES.

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Background: Mammalian cardiomyocytes withdraw from the cell cycle during postnatal development, which significantly limits the capacity of the adult mammalian heart to regenerate after injury. Therefore, proliferation of cardiomyocytes is promising strategy for treatment of heart disease, such as myocardial infarction. MicroRNAs are a short, non-coding, single stranded RNA that are 21-25 nucleotides in length and involve in the balance of a variety of developmental events, such as proliferation and differentiation, in many types of cells. Objective: To identify microRNAs that can induce proliferation of cardiomyocytes. Results: We screened a library of human microRNAs in primary culture of neonatal rat cardiomyocytes using BrdU incorporation assay. We successfully discovered two microRNAs, miR-148a and miR-152, of which seed sequences are identical, showed the highest induction of BrdU incorporation. Forced expression of miR-148a/152 in cardiomyocytes caused significantly higher percentage of Ki-67 positive cells (16.9±3.9%, 46.5±5.2%, 48.3±8.0% in control, miR-148a and miR-152-treated cells, respectively) and phospho-histone H3 positive cells (3.3±0.5%, 8.1±1.0%, 8.9±1.7% in control, miR-148a and miR-152-treated cells, respectively) at 48h after microRNA treatment, which indicates cell cycle reentry and mitosis can be proceeded in cardiomyocytes. Subsequently, in order to determine whether miR-148a/152 can induce cell cycle progression *in vivo*, we injected the adenoviruses harboring miR148a/152 into the left ventricle of the adult rat hearts. miR-148a/152 showed the remarkable expression of Ki-67 positive cardiomyocytes 4 days after infection (0.84±0.36%, 4.15±0.75%, 2.65±0.64% in control, miR-148a and miR-152-treated hearts, respectively. Conclusion: miR-148a/152 can induce cell cycle progression and mitosis in post-mitotic cardiomyocytes. Our results provide new insights into the development of therapeutic strategies for treatment of heart disease by induction of cardiomyocyte proliferation.

Poster Board Number: T-2030

FETAL EPIGENETIC MODIFIERS STIMULATE CARDIOMYOCYTE REGENERATION AND PROTECT FIBROSIS IN MAMMALIAN/AMPHIBIAN MODELS.

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Adult fishes and amphibians have high potential for heart regeneration, but major mammailans' model, mice/rat lose their potential by 7~10 days after birth. To understand this plasticity, we focused on several epigenetic factors which have unique expression patterns both in embryogenesis and in cardiac regeneration. SWI/SNF-cardiac BAF type chromatin remodeling factors and histone regulators were strongly up-regulated within 12 hours after resection of ventricle heart in neonatal mice and axolotl, and keep these expression for one week during regeneration. Whereas in adult rodents, no-expression of BAFs is disrupted within postnatal 10 days, indicating that they act as early response factors during heart regeneration and the most important key factors for regeneration in amphibian as well as in neonatal mice. Stable expression of BAFs in adult heart (BAF-TG) prevented fibrosis in myocardial infarction (MI) experiments and led to regenerate their lost parts of heart by new cardiomyocytes, keeping heart function healthy. Suprisingly, *in vivo* ChIP and ChIP-seq analyses showed that the major cardiac contracted genes' promoters of cTnnt2, Myl7, Nppa, were still opened in BAF-TG adult mice and sevaral fetal cardiac gene were activated in the presence of SWI/SNF-BAFs. In addition, we screened out repressors for SWI/SNF-BAF, suggesting that these factors controls cardiomyocyte maturation and reduction of regenerative plastitcity. These data suggest that combination of active/repressive chromatin modifiyers are necessary to stimulate/lose the plasticity of regenerative response and keep early cardiac gene programs in mammal/amphibian heart regeneration.

Poster Board Number: T-2031

FUNCTIONAL CHARACTERIZATION OF STEM CELL-DERIVED CARDIOMYOCYTES USING AN AUTOMATED PATCH CLAMP SYSTEM

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The development of automated electrophysiology instruments in the past decade has revolutionized ion channel drug discovery and safety pharmacology by greatly increasing the throughput of patch clamp assays. This development enables pharmaceutical and biotechnological companies to employ direct electrophysiological determination of compound activity on ion channels, with much larger numbers of compounds, and at earlier stages of the drug discovery and development process. However, automated electrophysiology assays require large quantity of highly homogeneous cells, which often limits the application of current systems to recording from recombinant cell lines stably transfected with ion channel of interest, rather than primary cell preparations that are more physiologically relevant. As a step forward, recent advances in stem cell research have shown great promise in providing stem cell-derived human cells, of all types and in sufficient quantity, for preclinical studies. In this study we attempt to address the growing

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interest to combine the stem cell technology and high-throughput automated electrophysiology for ion channel drug development and safety assessment. Specifically, we analyzed the electrophysiological properties of stem-cell derived cardiomyocytes using an automated patch clamp system, in both voltage clamp and current clamp mode. The ability to record action potentials and to also study multiple native ion channel targets from the same cell has significantly increased the sophistication of experimentation. The electrophysiological data is also comparable to that obtained using conventional patch clamp recordings. In conclusion, the combination of stem cell derived- cardiomyocytes with an automated patch clamp system offers a powerful assay platform not only for faster assessment of compound efficacy but also for safety profiling of lead compounds in a more biologically relevant system.

Poster Board Number: T-2032

ES AND IPS CELL DERIVED CARDIOMYOCYTES IN CARDIOVASCULAR DRUG DISCOVERY,SAFETY PHARMACOLOGY AND AS DIESEASE MODELS IN CARDIAC DRUG DEVELOPMENT

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Drug induced arrhythmia of the torsade des pointes types has been the reason for the denial of approval of novel drug candidates. The aim of cardiac safety pharmacology is to detect undesirable pharmacodynamic drug effects within and above the therapeutic range. A special focus is on the identification of potential arrhythmogenic effects within the drug discovery chain. Areas covered: Here, the authors discuss the relevance of induced pluripotent stem (iPS) cell derived cardiomyocytes for safety pharmacology. The technology of obtaining functional cardiomyocytes from somatic cells of healthy donors and patients with inherited diseases is the basis for diverse disease models in multi-level safety pharmacology screening. Here we compare the properties of iPS cell derived cardiomyocytes, ES cell derived cardiomyocytes with a non mammalian primary cardiomyocyte based assay and heterologous expression systems. We compare results from cardiac tissue recordings and Langendorff hearts with our datasets obtained from a variety of stem cell derived cardiomyocytes for a panel of reference compounds. The data presented do not just focus on prolongation of the ventricular action potential or the QT interval in the ECG, but also include an analysis of pro-arrhythmic events.

Poster Board Number: T-2033

NOTCH1 REGULATES CARDIOVASCULAR PROGENITOR DIFFERENTIATION

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Cellular decision making of self-renewal or differentiation arises from integration of intrinsic and extrinsic signals. Notch is an evolutionarily conserved transmembrane protein with critical roles in numerous cell-fate decisions. The Notch pathway is prominent among those known to regulate stem and progenitor development in multicellular organisms from flies to vertebrates. We recently showed in mice that conditional knockout of Notch1 in cardiovascular progenitor cells (CPCs) leads to a marked expansion of CPCs, but causes early embryonic lethality due to cardiac morphogenetic defects, making it difficult to address the fate and cell-autonomous role of Notch1. To overcome this problem, we have derived embryonic stem (ES) cells from Notch1-conditional mutant embryos with fluorescent markers. We have used these ES cells to study CPC-autonomous role of Notch1 in cardiac differentiation and discovered a critical role of Notch signaling for the lineage-specific differentiation of CPCs.

Poster Board Number: T-2034

DE-DIFFERENTIATED FAT AS A CELL SOURCE FOR CARDIOVASCULAR TISSUE ENGINEERING

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The source and availability of the cells for tissue engineering (TE) is critical. Ideally, the cells used for TE should be autologous and have the capacity to proliferate and differentiate in vitro, in a manner that can be reproducibly controlled. Although progress has been made towards differentiating embryonic stem cells as well as iPS cells to specific cell lineages, the efficiency is often poor and the number of cells generated is not efficient for therapeutic application. In the search for an ideal cell source for cardiac repair, we propose to study whether pluripotent adipocyte-derived mesenchymal stem cells, which we refer to as de-differentiated fat (DFAT) cells, can be a potential cell source for cardiovascular TE. Materials and Methods: Mouse DFAT cells were isolated in large numbers from fresh subcutaneous fat and cultured in DMEM supplemented with 20% fetal bovine serum and 0.5% of antibiotic-antimycotic solution as describes before. C57BL6/J mice, 25-30 grams, were subjected to experimental myocardial infarction (MI) and assigned to one of the following groups: 1) DFAT cells in DMEM supplemented with 1% Methylcellulose, 2) DMEM supplemented with 1% Methylcellulose (control), 3) DMEM alone (control), and 4) sham. The left anterior descending artery (LAD) was permanently ligated proximally with a suture. After the LAD ligation, once the heart rate stabilized, a cocktail of 500,000 cells in 10 µl DMEM supplemented with 1% Methylcellulose, or for controls without cells, were injected into the border zone of the infarcted area. Successful ligation was confirmed by decreased myocardial ejection fraction from ~70% before and 40-50% after ligation as measured by echocardiography. MI was further confirmed by the presence of collagen-rich infracted areas in Masson's trichrome-stained heart sections. Preliminary results: It has been shown that DFAT cells develop networks of beating cardiomyocyte (CM)-like cells after 3 weeks in culture. These cells express cardiac-specific markers such as Nkx-2.5, troponin C and connexin 43. Intracellular Ca2+ transients, action potentials, and contractions are observed in these networks. In addition, the DFAT cells have the capacity to undergo smooth muscle (expressing SM-actin, caldesmon, calponin and SM-myosin) and endothelial cell differentiation (expressing CD31 and CD144), which contract in response to carbamoylcholine chloride and form tube structures, respectively. Our preliminary results from injection of GFP-DFAT cells into the MI area in mice show the presence of the cells in the vascular structures and the heart muscle in the infarct zone up to 8 weeks post MI. This suggests that DFAT cells may support the formation of new vessels and cardiac cells in vivo. Our preliminary data from left ventricular catheterizations indicate an improved cardiac contractility and relaxation up to 8 weeks post MI, as compared to controls. However, additional studies are required to assess whether the DFAT cells had a beneficial effect on the myocardial function. Our expectation from this study is that the results will contribute to improve cardiac function in mouse models of cardiac disease and ultimately in patients. Our findings, will lead

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to new strategies to propagate the desired cell population on a large scale, essential for further development of stem cell based therapies.

Poster Board Number: T-2035

MOUSE MESENCHYMAL STEM CELLS DERIVED FROM INDUCED PLURIPOTENT STEM CELLS DEMONSTRATED MYOCARDIOGENESIS POTENTIAL

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Introduction : Aging adult donors-derived mesenchymal stem cells (MSCs) are functionally impaired in cardiac regeneration. Although MSCs derived from early embryonic stage such as embryonic stem cells or induced pluripotent stem cells (iPSCs) have great potential of proliferation and differentiation in previous studies, therapeutic capacity of mouse MSCs derived from iPSCs remains elusive. This study is therefore aimed to derive and isolate mouse MSCs from iPSCs for myocardiogenesis potential study in experimental myocardial infarction models. Methods : Mesenchymal-like stem cells were isolated from mouse iPSCs by removing feeder cells and leukemia inhibitory factor (LIF). MSCs were enriched by conditioned medium with basic fibroblast growth factor (FGF2) and epidermal growth factor (EGF) supplements before phenotypical characterization of MSCs by flow cytometry, utilizing phycoerythrin (PE)- conjugated antibodies against CD34, CD44, CD73, CD90, CD133, Oct4 and TRA-1-60. Multipotent differentiation studies of MSCs, including adipogenesis, osteogenesis and chondrogenesis have been carried out. Purified MSCs were further engaged to a recombinant cocktail formulated with transforming growth factor-beta 1, bone morphogenetic protein-4, activin A, retinoic acid, insulin-like growth factor-1, fibroblast growth factor-2, alpha-thrombin, and interleukin-6. Derived cardiopoietic mouse MSCs were transplanted into myocardial infracted murine model. Results : The iPSC derived cells were negative for hematopoietic markers CD34 and CD133, pluripotency markers Oct4 and TRA-1-60, while being positive for mesenchymal markers, CD44, CD73 and CD90. These cells were further induced into osteocytes and chondrocytes under differentiation conditioned medium. High expression of homeobox transcription factor Nkx 2.5, ventricular myosin light chain MLC-2V, and sarcomeric alpha-actinin were observed in cardiopoietic mouse MSCs. Conclusion : Mouse iPSCs derived MSCs demonstrated myocardiogenesis potential induced by a recombinant cocktail of growth factors. They hold potential capacity to attenuate ischemic cardiomyopathy.

Poster Board Number: T-2036

COMPREHENSIVE SCREENING OF CELL-SELECTIVE PEPTIDES AND ITS APPLICABILITY TO VASCULAR IMPLANT SURFACE MODIFICATION

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To reduce risks in long-term impolants, especially with vascular implants, tissue engineering technology to design effective scaffold property for managing proper regeneration of surrounding cells is important. The design strategies of the medical implant surface had been mainly focused to provide inhibitory effect on cell adhesion and growths. However, since such inhibitory effect on cells is against the regeneration process, those implants designed with inhibitory function have not yet achieved to eliminate risks of side-effects. To breakthrough such limitations of medical implant surface design, our natural regenerative mechanism has a great potential to be investigated. Etracellular matrix (ECM) is one of the most effective biomaterials to enhance the cellular regeneration. In spite of their promising effect, ECM itself is difficult for industrial production with stable and safe product quality. Animal-drived pathogenic risks are one of the main considerations. To mimic the function of ECM as a regeneration enhancing material, taken together with the feasibility of industrial production, we have been investigating short peptides that provide cell-selectivity as a scaffold modification biomaterial. By combining in silico peptide screening algorithms and high throughput peptide array-based cell assay technique, we have reported some clusters of short peptides that can selectively control the adhesion of different types of cells. Compared to ligand-type peptides, our proposing cell selective peptides modifies the implant material surface by their property. In other words, such peptides are the candidate blocks to interpret the properties of ECMs, and to be replaced by analogue molecules that can be more effective to be manufactured as implant material. We here show the effective screening strategies and results of such cell-selective peptides, and its applicability to madical implants by *in vivo* study of artificial vessel. We especially focus on conroling the selectivity of cells that critically affect the vascular implant sustainability, such as endothelial cells, smooth muscle cells, and mesenchymal stem cells.

Poster Board Number: T-2037

INDUCTION OF EPICARDIAL PROGENITOR CELL PROLIFERATION AS A REGENERATIVE MEDICINE APPROACH FOR ENDOGENOUS CARDIAC REGENERATION AND REPAIR

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Post-myocardial infarction cardiac dysfunction and heart failure remain a medical challenge as current therapies do not address the fundamental pathology, i.e. loss of functional cardiomyocytes. Our goal is to identify and develop a phenotypic screening assay for small or large molecules that enhance endogenous regenerative capacity, leading to efficient restoration of cardiac function post-myocardial infarction. Epicardial progenitor cells have been shown to play a major role in both heart development and repair of the myocardium. They are activated, although insufficiently, in adult hearts after injury and undergo an epithelial-to-mesenchymal transition that is regulated by expression of Wilm's Tumor protein 1 (WT-1). Taking the population of human epicardial progenitor cells that are WT-1(+), we assessed the basal proliferative capacity of several different donors in a kinetic imaging assay. We then tested a set of compounds from the literature that have been shown to have pro-proliferative effects in stem/progenitor cell populations such as 6-bromoindirubin-3′-oxi. This allowed us to determine the optimal window where a proliferative increase could be seen above basal levels. To enable screening of compounds at high throughput we further developed an endpoint imaging assay, we stained the cells with WT-1, Ki-67, and 5-ethynyl-2′-deoxyuridine (EdU) with Hoescht 33342 as a nuclear counterstain. To test the robustness of the assay, we treated the cells with an expanded set of compounds drawn from the literature including neuregulin-1, periostin, lithium chloride, thymosin β4, and VEGF. After assay validation, we moved into screening with a larger number of compounds including sets

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where the mechanism of action is known, which will aid in later deconvolution. Here we apply high-content phenotypic screening to assess the proliferation of epicardial progenitor cells following treatment with small molecules. This assay has the potential to be used as the basis for proliferation screens in other progenitor cell types.

Poster Board Number: T-2038

DYNAMIC MATRICES AND EMBRYONIC STEM CELLS IMPROVE CARDIAC OUTCOMES: A POTENTIAL MYOCARDIAL INFARCTION THERAPY

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Tissue-specific elasticity arises from developmental changes that occur in the environment over time, e.g. ~10-fold myocardial stiffening from E3 to E10 in the chick embryo. Recently, we have shown that pre-cardiac mesodermal cells plated on top of a thiolated hyaluronic acid (HA) hydrogel engineered to mimic this time-dependent stiffening improves cardiomyocyte maturation compared to cells on static compliant matrices. Here we examine in vitro and *in vivo* cell-matrix interactions, the effect of dynamic mechanical cues on the differentiation and development of mouse embryonic stem cells (mESCs), and *in vivo* biocompatibility. Improved embryonic cardiomyocyte and ESC distribution and viability were observed when cells were encapsulated and bound to immobilized, thiolated gelatin conjugated to the HA matrix. While mechanical cues alone are insufficient to efficiently induce cardiogenesis, a combination of growth factors (e.g. BMP4, Activin A, DKK-1) and mechanics can enhance maturation of mESC-derived cardiomyocytes. mESC-derived cardiac progenitors most affected by developmentally-relevant mechanical cues are those that naturally observe such variation *in vivo*. Though not toxic to cells in culture, we assessed HA's local and systemic biocompatibility via subcutaneous and intramyocardial injections. Prior to assembly, HA was injected into Sprague-Dawley rats and samples were removed over a post-injection time course and subject to histological, immunological and mechanical analysis. Histological analysis shows minimal infiltration of host cells and capsule formation for subcutaneously injected rats, indicating a limited local immune response. Hematological analysis shows no significant systemic immune response was elicited in pre- vs. post-injection animals compared to controls. Most importantly, atomic force microscopy (AFM) analysis of samples from subcutaneous injections demonstrates dynamically increasing hydrogel stiffness over time similar to that previously found in vitro. When injected intramyocardially, host cells begin to actively degrade HA within 1 week post-injection and lay down matrix, nearly replacing the gel with host tissue by 1 month. These data indicate that when ESC-derived cardiomyocytes are co-injected with HA, vascularization is likely to occur, supplying nutrients to differentiating ESCs. Altogether, we find that HA is a viable source for therapeutic use in treatment of myocardial infarction, and could enhance cardiomyocyte differentiation *in vivo*.

Cancer Cells

Poster Board Number: T-2041

FASCIN REGULATES BREAST CANCER STEM CELL DIFFERENTIATION AND FUNCTION.

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Despite great success in treating patients with localized tumor disease, treatment of metastasized tumor remains restricted. This restriction has been attributed to the fact that most chemotherapeutic remedies target bulk of differentiated cancer cells sparing cancer stem cells, which are believed to be responsible for tumor relapse and metastasis. The molecular pathway that regulates the maintenance and function of cancer stem cell pool within the tumor remains largely unknown. We have previously demonstrated that the expression of actin-bundling protein (fascin) in breast cancer cells regulates various metastasis-associated genes resulting in increased metastasis and shorter survival. Here, we used gain and loss of function approaches to examine whether fascin play a role in regulating cancer stem cell phenotype and function in breast cancer cells. Fascin was knockdown in MDA-MB-231 cell and expressed in T47-D cells, which are breast cancer cell lines positive and negative for fascin, respectively. Results showed that fascin expression was strongly associated with increased CD44 and reduced CD24 expressions, a phenotype consistent with more stem cell-like breast cancer cells. Fascin regulated breast cancer cell self-renewal capacity as indicated by decrease ALDH and Notch-1 activities when fascin was knocked-down. Furthermore, when seeded in low attachment culture dishes, fascin-positive cells formed more mamospheres than the fascin-knockdown cells. Most importantly, fascin-positive cells are more resistance to apoptosis when exposed to chemotherapeutic agents. Altogether, our data support the existence of small subpopulation in breast cancer cells with a stem cell characteristic and fascin play a key role in regulating this cancer stem cell population.

Poster Board Number: T-2042

ERBB/NF-KB SIGNALING REGULATES BREAST CANCER STEM CELL-LIKE PROPERTIES

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Cancer stem cells, a small proportion of heterogeneous tumor cells, can self-renew and simultaneously produce differentiated tumor cells with strong proliferative activity, and therefore are responsible for tumorigenesis. Although it is important to target cancer stem cells for treatment of cancer patients, it has been difficult to find appropriate target molecules because the mechanisms by which cancer stem cells maintain their ability remain obscure. Here, we identified a molecular mechanism that regulates breast cancer

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stem cell (BCSC)-like properties. We found that heregulin (HRG), a ligand for ErbB3, induced mammosphere formation in breast cancer stem cells (BCSCs) as well as in breast cancer cell lines. HRG-induced mammosphere formation was reduced by treatment with inhibitors for phosphatidyl inositol 3-kinase (PI3K) or NF-κB and by expression of IκBα-Super Repressor (IκBαSR), a dominant-negative inhibitor for NF-κB. Moreover, the overexpression of IκBαSR in breast cancer cells inhibited tumorigenesis in NOD/SCID mice. Furthermore, we found that the expression of IL8, a regulator of BCSC self-renewal, was induced by HRG through the activation of the PI3K/NF-κB pathway. These findings illustrate that HRG/ErbB3 signaling appears to maintain BCSC properties through a PI3K/NF-κB pathway in human breast cancer.

Poster Board Number: T-2043

MIR200C THERAPY TARGETING BREAST TUMOR STEM CELLS AS A TREATMENT FOR AGGRESSIVE BREAST CANCER

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Discovery of prominent molecular features in aggressive breast cancer that can be used to predict patient prognosis and/or targeted for therapy is essential. Deficiency or mutation of p53 is a hallmark of aggressive breast cancer (e.g. triple-negative breast cancer) which manifests high tumor grade, poor prognosis, and cancer recurrence. Our previous study *(Chang et al., Nature Cell Biology, 2011)* reveals that tumor suppressor p53 plays a critical role in regulating both EMT (epithelial- mesenchymal transition) and stem cell properties through transcriptional activation of microRNA-200c (miR-200c). Loss or mutation of p53 in human mammary epithelial cells leads to decreased expression of miR-200c, activated EMT program and enhanced undifferentiated stem cell population. Loss of miR-200c expression significantly marks breast cancer samples deficient in the functional p53, and predicts poor tumor differentiation as well as poor prognosis in patients. Notably, restoration of miR-200c suppresses gene targets that mediate EMT and stemness properties, which promotes epithelial cell phenotype and enforces differentiation of human and murine mammary tumor stem cells *in vitro*. A nanoparticle based-miR200c delivery system that targets p53-deficient mammary tumors *in vivo* is currently under test. Together, this study provides a novel therapeutic application of miR-200c which modulates differentiation of breast tumor stem cells to treat aggressive breast cancer or even a broad spectrum of cancers with p53-deficiency.

Poster Board Number: T-2044

REGULATION OF BREAST CANCER STEM CELLS BY SPHINGOSINE-1-PHOSPHATE

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Growing evidence suggests that tumors are organized in a hierarchy of heterogeneous cell populations and are formed and maintained from a small population of stem/stem-like cells, known as "cancer stem cell (CSC) model". CSCs are defined on the basis of characteristics such as high tumorigenicity, self-renewal, and differentiation that contribute to heterogeneity. Aldehyde dehydrogenase (ALDH), which is a detoxifying enzyme responsible for the oxidation of intracellular aldehydes, has been recently used as a functional marker for identification of CSCs. ALDH-positive cell population from breast tissue can be used as a predictor of clinical poor outcome in breast cancer patients. However, growth regulation of CSCs was not fully understood. In the present study, we searched intracellular lipids which increase the size of ALDH-positive cell population in breast cancer cells. We found that sphingosine-1-phosphate (S1P) increased the size of ALDH-positive cell population and expression levels of stem cell markers. To examine whether the effect of S1P is mediated through its receptor, we tested the effects of S1P receptor (S1PR) antagonists on the ALDH-positive cell population. The effect of S1P was inhibited by the S1PR3 antagonist CAY10444, not by the S1PR2 antagonist JTE-013. Since S1PR3 receptor is coupled with Gi and Gq proteins, we examined the subtype of G protein. Pertussis toxin, which is known to inhibit Gi signaling, inhibited the effect of S1P. Real-time PCR revealed that ALDH-positive cell population showed higher expression level of S1PR3, compared with ALDH-negative cells. To further investigate whether the effect of S1P is involved in a stem cell-dependent pathway, we examined the Notch, Wnt and Hedgehog signaling. We found that S1P induced an expression of Notch target gene Hes1. In contrast, S1P did not induce Wnt and Hedgehog targets. Furthermore, DAPT, which inhibits cleavage of activated Notch receptors by gamma-secretase and thereby prevents Notch signaling, reduced the S1P-induced Hes1 expression and the increase in ALDH-positive cell population. The Wnt inhibitor (PNU-74654) and the Hedgehog inhibitor (Cyclopamine) did not affect the effect of S1P. These data suggest that S1P induces expansion of breast CSCs via S1PR3, Gi, and Notch-dependent pathway.

Poster Board Number: T-2045

THE HIPPO TRANSDUCER TAZ CONFERS CANCER STEM CELL TRAITS ON BREAST CANCER CELLS DOWNSTREAM OF EPITHELIAL-TO-MESENCHYMAL TRANSITION AND THE DEREGULATION OF THE CELL POLARITY DETERMINANT SCRIBBLE

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Cancer Stem Cells (CSCs) are proposed to drive tumor initiation, heterogeneity and progression. Yet, our understanding of the cellular and molecular mechanisms that underlie CSC properties is limited. We present evidences indicating that TAZ, a transducer of the Hippo pathway, is a determinant of key biological traits of breast CSCs. TAZ is required to sustain self-renewal and tumor initiation capacities in cellular models of breast cancer progression. TAZ protein levels are stabilized in prospective CSCs, and gain-of-TAZ in non-CSCs induces them to adopt CSCs-like behaviors. We found that gene-signatures denoting TAZ activity are associated with molecular imprints of "stemness" in breast cancer patients' datasets. In agreement with the view that an increase in CSCs drives tumor progression in breast cancer (Pece et al., 2010), the proportion of TAZ positive cells is much more abundant in poorly-differentiated tumors than in well differentiated ones. Consistently, raising TAZ levels promotes the transition of experimentally induced tumors toward a less-differentiated status. At the molecular level, TAZ promotes self-renewal of CSC downstream of Epithelial-to-Mesenchymal Transition (EMT). This is caused by deregulation of the cell polarity determinant Scribble, which forms an endogenous complex with TAZ in nontransformed and tumoral mammary epithelial cells. Remarkably, loss-of-Scribble - or induction of EMT - disrupts the association of TAZ with the core Hippo kinases MST and LATS, allowing TAZ to escape phosphorylation by LATS and association to beta-TrCP ubiquitin ligase complex. This study thus links the

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CSCs concept to the Hippo pathway in breast cancer, and reveals a mechanistic basis of the control of Hippo kinases by cell polarity.

Poster Board Number: T-2046

PHENOTYPIC CHARACTERIZATION NORMAL AND CANCER STEM CELLS WITHIN THE DIFFERENT SUBPOPULATIONS OF BREAST CELLS

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Strong evidence supports the existence of stem cells in the normal breast. The phenotype of these cells has been characterized using several unrelated markers. Therefore, the overlap between these different cell populations and their relation to breast cancer cells is not well known. To study the breast cells subpopulations we isolated single cells from breast tissue and analyzed these cells by multi-parametric flow cytometry. Results show that breast mesenchymal cells had the phenotype of CD49fneg/Ep-CAMneg/CD44high/CD24neg. The luminal epithelial cells were Ep-CAMhigh and were distributed between differentiated MUC-1high/CD49f-neg luminal cells and a progenitor CD49f+ luminal cells. The basal epithelial cells were Ep-CAMlow/CD49f+ and were distributed between differentiated CD10neg and progenitor CD10+ basal cells. All tested stem cell markers were restricted to CD49f+ cells although some of them did not overlap. for example, the previously described stem cell markers ALDHhigh and CD10 were exclusively expressed by cells in Ep-CAMhigh/CD49f+ luminal progenitor and basal Ep-CAMlow/neg/CD49f+ cell respectively. Other stem cell markers were distributed among the different subpopulations of CD49f+ breast cells as ABCB1 and ABCG2 were mainly Ep-CAMlow while CD133, CD184 (CXCR-4) and CD117 (c-kit) were Ep-CAMhigh. In comparison, cancer cells were mainly Ep-CAM+, consistent with luminal origin of breast cancer. Interestingly, the majority of breast cancer samples (~85%) had tumor cells that were mainly MUC-1high/CD49fneg a phenotype similar to differentiated normal luminal cells. In addition, majority (70%) of breast cancer samples also had a population of CD49f+ cells constituting 12-87% of breast cancer cells. Further look into the different subpopulations of cancer cells shows that while CD44high were distributed between CD49f+ and CD49fneg cells, ALDHhigh cells were mainly CD49f+ . In conclusion, our data identify different subpopulations of normal and breast cancer cells, an important contribution to the understanding of mammary gland development and therapeutic targeting of breast cancer cells.

Poster Board Number: T-2047

BREAST CANCER STEM CELLS: TARGETED THERAPY FOR BREAST CANCER

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Simple BRCA screening is insufficient for 'event-free survival' as breast cancer is clinically and pathologically an extremely heterogeneous disease. Targeting Breast Cancer Stem Cells (BCSCs) present in bone marrow and breast tissues is a lucrative alternative. Identification of BCSCs is salient aspect of our research. Invasive and mesenchymal property of BCSCs with CD44+/CD24low/ALDH1+ phenotype has made them a promising target for eliminating metastatic capacity of primary tumors. We hypothesize that ability to therapeutically attack stem cell hinges upon identifying unique targets like cell surface markers and this will decide development of specific target therapies. A total of 10 early chemo-naive patients with biopsy proven triple-negative metastatic breast cancer in the age group of 18-36 yr.s (mean age 28 yr.s) were selected randomly and tested for CD44/CD24 cell surface markers following immunosorting using magnetic cell sorter and immunophenotyping by flowcytometric analysis. Isolated BCSCs were cultured for in vitro drug sensitivity towards platinum, anthracycline and docetaxel. Correlation was drawn between cell differentiation, % of stem cells and drug response. Accordingly chemotherapy was designed for a particular patient. % of BCSCs in pre- and post-chemotherapeutic condition was further compared. We have detected BCSCs in 90% of cases. Among positive samples, 89% patients showed platinum sensitivity and rest were found to be anthracycline sensitive. No sensitivity to docetaxel was observed. In lieu of this, cisplatin was applied *in vivo* and % of BCSCs came down to 6.58% from initial 11.16% (for a representative case). Thus primary aim to target BCSCs at the onset of tumors in breast cancer patients to control metastasis and relapse of disease was somewhat obtained. We further plan to correlate ratio of selected markers present in patients in pre- and post-chemotherapeutic condition with time to recurrence, mortality, morbidity and progression-free survival. Finally, if no BCSCs prevail after chemotherapy, then patients would be kept under observation and if traces are found, we would proceed to stem cell replacement.

Poster Board Number: T-2048

GENERATION OF A NOVEL CANCER STEM CELL-BASED TRANSGENIC MICE OVEREXPRESSING OCT4B1 VARIANT AS A CANCER MODEL

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Since the first gene transfers into mice were successfully executed in 1980, transgenic technologies offered new approaches for life science researches. Fitting the current biotechnology demands, transgenic methods were dramatically used for the past two decades as outstanding tools for *in vivo* target validation, drug optimization, and pre-clinical studies. Transgenic animals can provide investigators with valuable *in vivo* models to study biology and etiology as well as novel approaches in the early detection and targeted therapies of defined cancers without arising strict ethical concerns and restrictions. A growing body of recent studies supports the notion that both tumor initiation and sustaining rely on only a small subset of cells within a tumor, termed tumor-initiating or cancer stem cells (CSCs). Few investigators believe that CSCs reside in different organs and grow upon ideal microenvironment. However, there is now a theory that stem cells which reside in many adult tissues are not only responsible for the normal reparative and regenerative processes, but are also considered to be a prime target for genetic and epigenetic changes, culminating in abnormal conditions leading to generation of CSCs. Whatever the origin of cancer is considered, the importance of CSC research lies on the improved understanding of cancer biology and its potential roles to provide new approaches in cancer early detection and targeted personalized treatment. Recent studies have revealed specific expression of stem cell proteins in cancer cells with it. Oct4, a key regulator and specific marker of totipotent cells *in vivo* and in vitro, is critically involved in the self-renewal and pluripotency networks of ES and EC cells. Human oct4 gene potentially encode three

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different spliced variants; OCT4A, OCT4B and OCT4B1. These have different expression pattern. It was reported that novel alternative variant, OCT4B1, sharply down-regulated during the course of differentiation of human ES/EC cells and suggested correlation between OCT4B1 and pluripotent/ undifferentiated state of human ES/EC cells. Moreover, it is determined that there is a relationship between OCT4B1 expression and apoptosis and carcinogenesis. Thus, generation of the transgenic mice with CSCs in their tissues can provide researches with valuable *in vivo* models for studying the breast cancer biology, etiology, early diagnosis, and targeted therapies. To this end, we could design and produce transgenic mice for the first time with CSCs under inducable conditions. Our novel CSC-based cancer model may open new insights to improve our understanding of cancer biology and development of more efficient approaches in its early diagnosis and targeted therapies.

Poster Board Number: T-2049

NOTCH SIGNALING IS UPREGULATED IN THE CHRONIC PHASE OF CHRONIC MYELOID LEUKEMIA

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Notch signalling is critical for haemopoietic stem cell self-renewal and survival.Chronic Myeloid Leukaemia (CML) is a stem cell disease characterised by the presence of the Philadelphia (Ph) chromosome, and subsequent expression of the BCR-ABL oncogene. The well established role for Notch signalling in human T-cell acute lymphoblastic leukaemia (T-ALL) and the reported interaction between Notch and ABL in different developmental contexts in *Drosophila* raise the possibility that Notch signalling may be dysregulated in CML. Therefore, the aim of the study was to investigate whether Notch signalling is altered in CML and to study possible crosstalk between Notch signalling pathway and BCR-ABL in CML. The gene expression patterns of all four human Notch genes and the Notch target gene HES1 were studied in CD34+ stem and progenitor cells isolated from CML patients. Poly-A PCR followed by real time PCR analysis was used to quantitate gene expression levels in comparison with levels in equivalent populations isolated from normal bone marrow (NBM). The expression of Notch1 receptor protein levels expressed on the cell surface was also investigated by flow cytometry. Results showed an up-regulation of Notch1 and Notch2 genes and the target gene Hes1 on the most primitive CD34+ Thy+ subset of CML CD34+ cells as compared with NBM. In addition, Notch1 receptor protein was expressed in distinct lymphoid and myeloid progenitors within the CD34+ population of CML cells. These results suggest that Notch signalling may be highly activated in CML primitive progenitors. To investigate the possible crosstalk between Notch and ABL in vitro human cell line model systems were assessed as possible models to study the interactions between Notch and ABL signalling and the FACS based P-crkl assay was optimised as a rapid method to assess ABL activity. The data showed that K562 and ALL-SIL cell lines are sufficient model systems to investigate the cross-talk between the Notch and ABL signalling pathways. The imatinib induced inhibition of ABL activity in K562 and ALL-SIL cells resulted in significant up-regulation of Notch activity as assessed by Hes1 expression. Similarly, GSI inhibition of Notch signalling in K562 cells resulted in hyperactivation of ABL kinase activity as assessed by P-crkl levels. The antagonistic relationship between Notch and ABL signalling observed in cell lines were further confirmed in CD34+ cells from chronic CML patients. Treatment of CD34+ CML cells with imatinib led to significant up-regulation of Notch activity whereas inhibition of Notch signalling with GSI in CD34+ CML cells resulted in increased ABL activity. It can be concluded therefore, that Notch signalling may be dysregulated in the chronic phase of CML. In addition, the data presented in this project demonstrate for the first time the cross-talk between Notch signalling and ABL signalling in cell line model systems as well as in primary CD34+ CML cells. Future work is required to address the possible mechanisms that underlie the findings observed here and to investigate the biological consequences of the interplay between Notch and ABL signalling in CML.

Poster Board Number: T-2050

ACUTE MYELOID LEUKEMIA STEM CELLS LACKING RAPTOR SELF RENEW BUT HAVE DEFECTIVE LEUKEMIA INITIATING CAPACITY IN MICE

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Although dysregulation of the mammalian target of rapamycin complex 1 (mTORC1) promotes leukemogenesis, it is unclear how mTORC1 affects the behavior of established leukemia. Here we investigated the roles of *Raptor*, an essential component of mTORC1, in mouse hematopoiesis and leukemia by using an *in vivo* tamoxifen-inducible deletion system. *Raptor* deficiency impaired granulocyte and B cell development but did not alter survival or proliferation of hematopoietic progenitor cells. In an MLL-AF9-driven acute myeloid leukemia (AML) model, *Raptor* deficiency significantly suppressed leukemia progression by causing apoptosis of differentiated, but not undifferentiated, leukemia cells. mTORC1 did not control cell cycle or cell growth in AML cells. Transplantation of *Raptor*-deficient undifferentiated AML cells in a limiting dilution revealed that mTORC1 is essential for leukemia initiation. Strikingly, however, a subset of AML cells with undifferentiated phenotypes survived long-term *in vivo* without mTORC1 activity. The reactivation of mTORC1 in those cells restored their leukemia-initiating capacity. Thus, AML cells lacking mTORC1 activity can self-renew as AML stem cells. The findings provide mechanistic insight into how residual tumor cells circumvent anti-cancer therapies and drive tumor recurrence.

Poster Board Number: T-2051

ANDROGEN ABLATION MITIGATES DEFECT OF B CELLS TO A PROSTATE CANCER AND INCREASE CANCER AND INCREASE SURVIVAL RATE OF TRAMP MICE.

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Androgen ablation is the most commonly used therapy for prostate cancer. However, the effects of androgen ablation on immune system, especially B cell distribution are not well understood. We have used transgenic adenocarcinoma mouse prostate (TRAMP) mice to characterize the B cell distribution in periphery blood and spleen upon androgen ablation. We found a decrease of the B cell population in wild type TRAMP mice compared to normal wild type mice. Furthermore, the B cells increased when in castrated TRAMP mice compared to the wild type TRAMP mice. Interestingly, we found an increase in immature B cells in the spleen of castrated TRAMP mice, which might be due to the resistance to apoptosis during B cell

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maturation. Our data suggest that androgen ablation might play an important role in the regulation of B cell tolerance.

Poster Board Number: T-2052

ELEVATED ADAR1 EXPRESSION DRIVES CELL FATE DETERMINATION AND PERSISTENCE OF LEUKEMIA STEM CELLS IN CHRONIC MYELOID LEUKEMIA

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Cancer stem cells (CSCs) are a specific population of therapy-resistant cancer cells that have acquired stem cell properties such as self-renewal, survival, and differentiation, and the ability to home to protective niches. Chronic Myeloid Leukemia (CML) is the first cancer that was proven to originate from a genetic abnormality, the Philadelphia BCR-ABL translocation. Research demonstrated that BCR-ABL tyrosine kinase was necessary and sufficient to initiate chronic phase (CP) CML, while additional mutations in an expanded progenitor population may result in leukemia stem cell (LSC) generation and blast crisis (BC) transformation. The molecular mechanisms driving malignant transformation of progenitors into therapeutically recalcitrant LSC in CML have remained elusive. Generation of self-renewing LSC has been linked to activation of transcriptional programs typical of primitive stem cell populations. We propose that transcriptomic diversity fueled by aberrant RNA editing might promote malignant reprogramming of CML BC progenitors, allowing them to persist and evade therapy. Cumulative whole transcriptome RNA sequencing, hematopoietic progenitor assay, serial transplantation, lentiviral overexpression and shRNA knockdown experiments demonstrate that a key RNA editing enzyme, adenosine deaminase acting on dsRNA (ADAR)-1 regulates LSC cell fate determination and self-renewal capacity of CML BC progenitors. During blastic transformation, BCR-ABL amplification in progenitors positively correlates with increased levels of the interferon responsive ADAR1 p150 isoform. Corresponding activation of RNA editing favoring A-to-G substitutions and differential expression of ADAR target genes were also observed. Hematopoietic progenitor assays demonstrate that lentivirally driven ADAR1 p150 expression enhances PU.1 expression and skews cell fate towards granulocyte-macrophage progenitors - the initiating LSC population in BC CML. Finally, lentiviral ADAR1 shRNA knockdown significantly impaired human CML BC LSC serial transplantation potential in immune compromised mice. Together, our data support a pivotal role of ADAR1 and RNA editing in myeloid cell fate determination and self-renewal potential of malignant progenitors that drive disease progression and therapeutic resistance in CML.

Poster Board Number: T-2053

CHARACTERISATION OF HUMAN PROSTATE CANCER STEM CELL COLONIES IN CELL LINES

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The clonogenic assay is an important tool in the study of normal and neoplastic stem cells and demonstrates heterogeneity within normal and tumour cell populations in terms of cell morphology and proliferative potential. This study used the clonogenic assay to characterise prostate cancer cell colonies. Prostate cancer cell lines form three morphologically distinct colony types *in vitro*, which were compared for long term serial colony forming, sphere forming efficiency, tumourigenicity and expression of stem cell/differentiation and proliferation markers. Type 1 colonies are large and consist of small tightly packed cells, whereas type 3 colonies are small and consist of large loosely packed cells. Type 2 colonies are larger than type 3 and consist of a mixture of small and large cells. Both type 1 and type 2 colonies are able to be serially cloned long term, form spheres and are tumorigenic in mice. Type 3 colonies cannot be cloned or form spheres. Type 1 colonies also have an increased Ki67 positive fraction compared to Type 2 and Type 3 and express the cancer stem cell markers CD44 and α2β1 integrin. Type 1 colonies were previously thought to be derived from stem cells and Type 2 from transit amplifying cells. In contrast, this study shows both Type 1 and 2 colonies contain cells with stem cell traits, but they differ in the proportion of these cells.

Poster Board Number: T-2054

INVSETIGATIOM OF OCT4-DERIVED PARACRINE EFFECT IN MODULATING CANCER STEM-LIKE PROPERTY IN COLORECTAL CANCER

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Cancer stem cells (CSC) or tumor-initiating cells (TICs) are subpopulation of tumor cells to be responsible for resistance to chemotherapy, progression as well as recurrence in a variety of cancers, including colorectal cancers (CRCs). Several evidences have been demonstrated the relationship between inflammation and tumorigenesis in CRCs and proinflammatory cytokines have been suggested to regulate preneoplastic growth during colitis-associated cancer tumorigenesis. Interleukin (IL)-32, a proinflammatory mediator, can induce the expression of various cytokines, such as tumor necrosis factor-alpha (TNF-alpha), IL-1beta, IL-6, and IL-8. Since these cytokines would activate several signaling pathway to promote cell growth or survival like JAK/STAT3 pathway. However, the potential role of cytokines in mediating the CSC-initiated tumor progression or recurrence in CRCs is unclear. In this study, we found Oct4 overexpression enhanced cancer stem-like property in colorectal cancer cell line. Microarray-based bioinformatics showed that higher expression levels of embryonic stem cell (ESC)-specific genes in Oct4-expressing colorectal adenocarcinoma cell line (HT29-Oct4). In addition, the overexpression of Oct4 enhanced stem cell properties, which was contributed by the cytokines like IL-8 and IL-32. Targeting IL-8 and IL-32 in HT29-Oct4 cell with specific antibodies blocked these tumorigenic effects of Oct4-overexpressing CRCs. Furthermore, xenograft tumorigenicity assay revealed that Oct4-overexressing CRCs also enhanced the metastasis potential *in vivo*, which could be totally blocked by neutralizing IL-32 and IL-8. These data demonstrate that the cytokines IL-32 and IL-8 play a role in regulating stem-like properties and promoting tumorigenesis in CRCs. This may provide a therapeutic strategy for the treatment of CRCs.

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Poster Board Number: T-2055

EVALUATING THE EXPRESSION OF SEVERAL SELF RENEWAL GENES IN COLON, PROSTATE AND BLADDER CANCER AND IN CANCER CELL LINES (LNCAP, HEPG2, HT-1376)

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Background: Uncontrolled self renewal plays a direct function in different types of carcinoma progression. The same molecular pathway that manages self renewal in normal stem cells also images to be appropriate by cancer stem cells in cancers. Here we examined the expression of self renewal regulatory factors such as Oct4, Nanog, Sox2, Nucleostemin, Zfx, Esrrb, Tcl1, Tbx3 and Dppa4 in several tissue samples of colon, prostate and bladder cancer and in cancer cell lines (Lncap, HepG2, HT-1376)
Material & Method: We used RT-PCR (Reverse Transcriptase Polymerase Chain Reaction) to examine the expression of these gene in cancer cell lines and in 20 tumor samples. Total RNA was isolated using the ISOGEN method. RNA integrity was checked with agarose gel electrophoresis and spectrophotometry. The expression of Oct-4 and Nucleostemin at the protein level was further determined using immunocytochemistry. Results:. Oct4, Nanog, Sox2, Nucleostemin, Bmi, Zfx, Esrrb, Tcl1, Tbx3 and Dppa4 were expressed in bladder, colon and prostate cancer tissues and cell lines. Oct4 and Nucleostemin proteins were expressed in both nuclear and cytoplasmic in cancer cell lines. No immunoreactivity was observed in negative controls, which were incubated in the absence of primary antibody. Conclusion: Collectively, our data confirmed the expression of Oct-4, Nanog, Sox2, Nucleostemin, Bmi, Zfx, Esrrb, Tcl1, Tbx3 and Dppa4 in cancer cell cell lines and cancer tissues and suggested that their expression can be used as potential tumor markers in diagnosis and /or prognosis of tumors. These results confirm the potential value of the cancer stem-cell theory in cancer therapy

Poster Board Number: T-2056

THE ACQUISITION OF CANCER STEMNESS IN COLON CANCER IS REGULATED BY THE STABILIZATION OF ATONAL HOMOLOG 1 PROTEIN.

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Background & Aims: Atoh1 is one of the essential genes for secretory intestinal epithelial cells (IECs) differentiation. However, Atoh1 protein is not expressed in colon cancer region, despite Atoh1 gene is equally expressed between normal colon and colon cancer region. So, we have reported that GSK3 on Wnt signal reciprocally regulates the proliferation and differentiation by the ubiquitin proteasomal proteolysis of β-catenin and Atoh1 in sporadic colorectal cancer (CRC). Moreover, APC deletion on colorectal carcinogenesis causes Atoh1 protein degradation by GSK3, resulting in maintaining the undifferentiated state of CRC. On the other hand, Atoh1 protein is expressed in the mucinous carcinomas (MC) of colon in which APC is normal. However, it remains unknown whether Atoh1 affects the form of MC. So, we aim to elucidate Atoh1 function in MC. Methods: We constructed mutant Atoh1 fused with mCherry that reconstitutes five Serines of GSK3 target site to Alanin (mCherry-5SA Atoh1). mCherry-5SA Atoh1 induced CRC cells were assessed the differentiation/stem characteristics by RT-PCR and immunofluorescence. Cell growth was evaluated by MTS assay. Cell cycle analysis was performed by Live Cell Imaging and FACS using fluorescent cell-cycle indicator, Fucci (Fluorescent Ubiquitination-based Cell Cycle Indicator) system. *In vivo* study, nude mice were inoculated with naive CRC cells and mCherry-5SA-Atoh1 cells. Subsequently Oxaliplatin or DMSO was administered biweekly. The characteristics of tumors were assessed by the differentiation/stem markers and chemoresistance. Results: mCherry-5SA-Atoh1 showed the stable expression in CRC cells. Atoh1 protein stabilization up-regulated both E-BOX and TCF4 dependent transcriptional activity, resulting in the expression of Mucin2 (mucinous marker) and Wnt target gene, Lgr5 (cancer stem cell marker). Moreover, mCherry-5SA-Atoh1 inhibited cell growth to suppress the cell cycle by the extension of G0/G1 time. Furthermore, mCherry-5SA-Atoh1 acquired the chemoresistance against Oxaliplatin to avoid the caspase-dependent apoptosis. Interestingly, Wild type Atoh1 (mCherry-WT-Atoh1) inducing CRC cells also acquired the chemoresistance against Oxaliplatin, because we found that Oxaliplatin stabilized Atoh1 protein by the inhibition of GSK3 kinase activity. *In vivo* study, Tumor constituted by mCherry-WT-Atoh1 inducing CRC cells grew up regardless of the treatment with Oxaliplatin, resulting in the Atoh1 protein stabilization and MC phenotype acquisition. Conclusions: Atoh1 protein stabilization might regulate the form of MC, including cancer stemness and chemoresistance that reflect poor prognosis of MC.

Poster Board Number: T-2057

REAL TIME IMAGING OF THE DYNAMICS OF CELL DEATH INDUCED BY THERAPEUTIC NEURAL STEM CELLS IN MALIGNANT BRAIN TUMORS

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Neural stem cells (NSCs) have recently been recognized as efficient delivery vehicles in a number of malignancies including the most aggressive brain tumor type: glioblastoma multiforme (GBM). Therefore, engineering NSCs to express tumor-specific cytotoxic reagents, such as tumor necrosis factor related apoptosis-inducing ligand (TRAIL), offers great potential in the treatment of GBMs. In this study, we engineered TRAIL-sensitive and -resistant GBM cells with fluorescence-based live-cell caspase-reporters and assessed their response to SC-TRAIL in real-time. We show that SC-TRAIL induces caspase-mediated apoptosis in GBM cells, which is correlated with their death-receptor (DR)4/5-expression levels, and the sustained release of TRAIL via NSCs has significant anti-tumor effects in vitro and *in vivo*. To target TRAIL-resistant GBMs, we have developed DR-reporters that offer an imaging-based screening platform to identify agents that can act in concert with SC-TRAIL. Utilizing both DR4/5 and caspase-reporters, we show that an HDAC inhibitor, MS-275, augments the response of TRAIL-resistant GBM cells to SC-TRAIL in vitro and *in vivo*. This study demonstrates the efficacy of a combination of real-time reporters of TRAIL-mediated apoptosis pathway in evaluating the fate of tumor-cells in response to SC-TRAIL therapeutics and may have clinical implications for cancer patients.

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Poster Board Number: T-2058

BONE MARROW CELLS TRAFFIC AND FUSE TO DEVELOPING COLORECTAL TUMOR CELLS GENERATING TUMOR INITIATING CELLS

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Background: Bone-marrow-derived-cells (BMDC) can traffic to injured organs, and participate in organ maintenance under certain inflammatory conditions. Subsequently, rare reports suggested that cancers could originate from BMDC. Given the fact that tumors generate an inflammatory environment, we hypothesized that BMDC participate in tumor maintenance. Materials & methods: We obtained BMDC and fresh tumors from patients enrolled in this study after proper consent, approved by the NIH Institutional Review Board. We irradiated severe immunodeficient mice (NSG) and performed bone marrow transplantation (BMT) using human CD34+ cells. After 4 weeks of recovery, we xenotransplanted subcutaneously human colon cancer cells isolated from fresh surgical specimens. In the testing experiment, tumor and BMDC cells carried two different lentivirus reporter genes. In the validating experiment, tumor and BMDC cells were followed using HLA markers, and in a sex-mismatch experiment, we used a Y-chromosome marker. When tumors developed, we extracted the tumors and sorted and tested the cells according to their origins. Results: We show that human BMDC, traffic to sites of developing colorectal cancers, fuse to cancer cells, and generate tumor initiating cancer cells. Fused cells expressed genetic markers both from tumor and BMDC cells, were CD45-negtaive and expressed CEA. We confirmed the FACS data using qRT-PCR, DNA sequencing and Karyotyping. Quantitative DNA analysis using SNP specific pyro-sequencing showed cells containing DNA derived from both BMDC and colon cancer cells. Karyotyping demonstrated that cells that expressed both colon cancer-and-BM derived HLA were tetraploid while cells that express colon cancer-derived-HLA alone were aneuploid but not tetraploid. Bone-marrow-tumor-fused-cells expressed the typical colon cancer marker CEA, and maintained the malignant phenotype over several generations. Averaged over multiple experiments fused cells comprised 14%-to-28% of the genetic marker bearing tumor cells. Importantly, testing the tumor initiating capacity of tumor cells versus BM-tumor-fused-cells, the BM-tumor-fused-cells have the exclusive capacity to initiate tumors with only 10 cells generating a more aggressive and less differentiated phenotype with high Ki67 expression tumors. Finally, BM-tumor-fused-cells have unique pluripotency gene signature up-regulating Sox-2 (>200 folds), MYC, Notch, Numb, CD44, CDH1, BMP1, CXCL12, FOXA, FGF1-3 and ALDH-1/2 . Conclusion: Similarly to normal tissue maintenance, this data suggest that tumor maintenance can be supported by BMDC. These findings have potential far-reaching implications to our understanding of tumor progression, maintenance and novel approached for cancer therapeutics.

Poster Board Number: T-2059

DIFFERENTIATION OF EPITHELIAL OVARIAN CANCER STEM-LIKE CELLS INTO MESENCHYMAL LINEAGE RESULTS IN CELLULAR HETEROGENEITY WHICH ENABLES TUMOR ENGRAFTMENT

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Ovarian cancers are heterogeneous and contain stem-like cells that are able to self-renew and are responsible for sustained tumor growth. Metastasis in peritoneal cavity occurs more frequently in ovarian cancer than in other malignancies, but the underlying mechanism remains largely unknown. We have identified that ovarian cancer stem-like cells (CSCs), which were defined as side population (SP), were present in patients' ascitic fluid and mesenchymally transformed cell lines, ES-2 and HO-8910PM. SP cells, which were sorted from both cell lines and implanted into immunocompromised mice, were localized to the xenografted tumor boundary. In addition, SP cells exhibited an epithelial phenotype and showed a distinct gene expression profile with reduced expression of cell adhesion molecules (CAMs),indicating that SP cells play an important role in ovarian cancer peritoneal metastasis. In contrast, non-SP cells exhibited a more mesenchymal phenotype and showed increased invasive potential than SP cells. The engraftment ability of SP cells in immunocompromised mice was greatly increased when SP cells were injected as a differentiated heterogeneous population containing non-SP cells. This heterogeneity was observed as an endogenous transformation viathe epithelial-mesenchymal transition (EMT) process. Inhibition of the EMT process by Snail1 silencing reduced the SP cell frequency, and affected their invasive capacity and engraftment. These findings illustrate the interplay between epithelial ovarian CSCs and the EMT exerts a link to explain tumor heterogeneity and is necessary for ovarian cancer maintenance, metastasis and progression.

Poster Board Number: T-2060

ELEVATED LEVELS OF SIALYLATED C-MET INDUCED BY FRUCTOSE REPLACEMENT ENHANCES METASTATIC POTENTIALS OF PANCREATIC CANCER STEM CELLS

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Existence of pancreatic cancer stem/initiating cells (CSCs) has been considered as a possible link for poor clinical outcomes of pancreatic ductal adenocarcinoma. It has been proposed by Otto Warburg that mitochondrial alterations in cancer cells as the driving force of tumorigenesis. Our initial work demonstrated ABCG2$^+$ CD24$^+$ CD44$^+$ pancreatic cancer subpopulation expressed self-renewal regulators (e.g. c-Met, Sox2) and characteristics of CSCs such as higher tumorigenicity and chemo-resistance. During differentiation of ABCG2$^+$ CD24$^+$ CD44$^+$ subpopulation, these cells were found to produce higher levels of lactate and had higher oxygen consumption rate. Strikingly, these cells can still produce ATP after adding

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mitochondrial ATPase inhibitor-oligomycin indicating these cells display plastic metabolic features. Glucose deprivation culture altered cell responses to first-line anti-cancer drug and further selectively outgrew ABCG2$^+$ CD24$^+$ CD44$^+$ subpopulation. Recent cohort studies the higher pancreatic cancer risk associated with high free fructose intake, we further revealed fructose replacement not only can electively outgrow ABCG2$^+$ CD24$^+$ CD44$^+$ subpopulation, but it also enhanced invasion and metastatic potential of ABCG2$^+$ CD24$^+$ CD44$^+$ subpopulation *in vitro* and in the orthotropic mouse model. Comparing with the tumor-engraft mice fed with normal diet, fructose-enriched diet (60% fructose) enhanced cancer progression and liver metastasis. Importantly, the enhancement in metastatic potential by fructose replacement was found to associate with upregulation of alpha 2,6-sialylation and evaluated levels of beta-galactoside alpha 2,6-sialyltransferase (ST6Gal-I). The knockdown of ST6Gal-I affected cell motility. RNA-seq and target proteomics further revealed c-Met is the substrate of ST6Gal-I. The loss of alpha2,6-sialic acid on c-Met in ST6Gal-I knockdown abolish cell motility of ABCG2$^+$ CD24$^+$ CD44$^+$ subpopulation. In conclusion, c-Met is required for self-renewal for pancreatic stem cells and CSCs. And our current work identified metabolic reprogramming can enhance metastatic potentials of pancreatic cancer stem cells by increasing functionality of c-Met via alpha 2,6-sialylation indicating sialylated c-Met is a potential target for metastatic cancer stem cells of pancreatic ductal adenocarcinoma.

Poster Board Number: T-2061

TARGETING MOUSE GLIOMA CANCER STEM CELLS USING CPG-SISTAT3

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Glioblastoma multiforme (GBM), an aggressive grade IV glioma, is known to have high probability of recurrence after therapeutic challenge. Several lines of evidence suggest that cancer-stem cells (CSCs; also called "tumor-initiating cells") underlie GBM recurrence and resistance to current therapies. Furthermore, glioma cancer stem cells suppress innate and adaptive immune responses while creating an immunologically environment that limits the therapeutic benefit of immunotherapy. It has been demonstrated that the Signal Transducer and Activator of Transcription-3 (Stat3) is critical for glioma tumor-initiating cells' stem cell-like phenotype and creating an immunosuppressive microenvironment. We recently developed a platform to deliver siRNA synthetically linked to CpG *in vivo*; so-called CpG-siRNA. It has been shown that CpG-*Stat3*siRNA may be used to initiate robust anti-tumor immune responses in multiple cancer types; including melanoma, colon carcinoma, and lymphoma. Here, we demonstrate a powerful approach to curb CSCs in glioma by using CpG-*Stat3*siRNA, shifting the phenotype of the glioma cancer stem cells *in-vivo* and *in-vitro*. This induced shift would allow for the glioma cancer stem cells to become sensitive to conventional treatment protocols. Additionally, we demonstrate how the CpG-*Stat3*siRNA might guard against glioma recurrence *in-vivo*. Finally, we show how silencing Stat3 may shift the glioma's immune microenvironment towards an anti-tumor response. Thus, we consider CpG-*Stat3*siRNA as a multi-pronged therapeutic against glioma by selectively targeting the resistant phenotype of the glioma tumor-initiating cells, as well as causing an anti-tumor immune response against glioma tumor cells. These findings may lead to a more effective therapy against glioblastoma multiforme. (Supported by the California Institute of Regenerative Medicine and the National Institutes of Health)

Poster Board Number: T-2062

EVALUATION OF CD44 VARIANT EXPRESSION AS A RECURRENCE MARKER OF GASTRIC CANCER AFTER ENDOSCOPIC SUBMUCOSAL DISSECTION

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Background: As for the treatment of early gastric cancer (EGC), endoscopic therapy is widely used and have shown a good prognosis, although some of EGCs often show metachronous recurrence after initial endoscopic treatment. Recently, cancer stem cells having a stem cell-like ability such as multilineage potential and the self-renewal potential in a cancer tissue has been identified, and it is suggested that they are linked to tumor recurrence and metastasis. CD44 has recently been identified as one of the cell surface marker associated with cancer stem cells in various solid tumors. In addition, it was reported that human gastrointestinal cancers with a high level of CD44 variant (CD44v9) showed an enhanced capacity of glutathione synthesis, defence against reactive oxygen species (ROS) and reduced activation of p38MAPK, a downstream target of ROS (Cancer Cell 19, 387-400, 2011). In the present study, we investigated whether the expression of CD44v9 could become the marker of the recurrence of the gastric cancer. Method: A case-control study was conducted to identify CD44v9 as a recurrence marker of gastric cancer after initial endoscopic submucosal dissection (ESD) at the Keio University Hospital (Tokyo, Japan) from February 2008 to February 2009 within a follow-up period (33.6±7.9 months). The study compared 12 cases who had undergone curative resections for EGC and recurrence was identified after initial ESD with 13 age- and sex- matched controls. The expression of CD44v9 and activated p38MAPK (phospho-p38) were evaluated in immunohistochemistry. Quantification of the proportion of CD44v9-positive area (CD44v9 IHC score) was calculated by ImageJ (US National Institutes of Health, Bethesda, MD). The clinical features of the patients, such as age, sex, body mass index (BMI), H. pylori infection, smoking history, and location and differentiation of the tumors were retrospectively obtained from medical records. Result: The average of CD44v9 IHC score were 1.47±0.58% (median 0.63%). We divided patients into two groups of CD44v9 higher group (n=13) and lower group (n=12) based on a cut-off level which was decided with 0.61% by ROC analysis (sensitivity 75.0%, specificity 69.2%). In the analysis of recurrence rate using the Cox proportional hazard model, recurrence rate was significantly higher in CD44v9 higher group in comparison with CD44v9 lower group (p=0.015). Furthermore, a reduced expression of phospho-p38 was identified in CD44v9 positive cells. Conclusion: In the CD44v9 higher group, recurrence rate was significantly high as compared with the CD44vp lower group. Our result suggest that the expression of CD44v9, which up-regulates intracellular ROS resistance, is one of major predictive factors for the recurrence in the EGCs.

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SUPPRESSION OF CANCER STEM-LIKE PROPERTIES IN GLIOBLASTOMA BY DELIVERY OF MICRORNA145 SHORT BRANCH PEI

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Glioblastoma (GBM) the most common primary brain tumor bearing dismal prognosis. CD133 has been considered a putative marker of cancer stem cells (CSCs) in malignant cancers, including GBM. MicroRNAs (miRNAs), highly conserved small RNA molecules, may target oncogenes and become a therapeutic strategy against cancer. However, the involvement of miRNAs in GBM-associated CSCs remained mostly unclear. Using miRNA/mRNA microarray analysis, we identified that miR145 (a tumor-suppressive miRNA) expression is inversely correlated with the levels of Oct4 and Sox2 in GBM-CD133$^+$ cells and specimens of malignant glioma. Consistently, we demonstrated that miR145 negatively regulates tumor growth of GBM by directly targeting Oct4 and Sox2 in GBM-CD133$^+$ cells. We therefore evaluated the treatment effect of polyurethane-short branch polyethylenimine-mediated miR145 (PU-PEI-miR145) on GBM-CSCs. PU-PEI-miR145 delivery in GBM-CD133$^+$ significantly inhibited their tumorigenic and CSC-like abilities, and facilitated their differentiation into CD133$^-$ non-CSCs. Furthermore, PU-PEI-miR145 delivery in GBM-CD133$^+$ effectively suppressed their expressions of drug-resistance and anti-apoptotic genes, and dramatically increased the sensitivity to radiation and chemotherapeutic drugs, including temozolomide. Finally, *in vivo* alone delivery of PU-PEI-miR145 significantly suppressed tumorigenesis, and synergistically improved survivals in orthotopic GBM-CD133$^+$-transplanted immunocompromised mice, combined with radiotherapy and temozolomide. Therefore, PU-PEI-miR145 is potential therapeutic approach for malignant brain tumors.

Poster Board Number: T-2064

ROLE OF A7 NICOTINIC ACETYLCHOLINE RECEPTOR IN CANCER STEM CELLS

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Epidemiological studies have suggested that cigarette smoking is related to increased cancer risk. Nicotine, a major component of cigarette smoking, is believed to be responsible for the pathogenesis of smoking-related malignancies. However, the mechanisms by which nicotine promotes cancer development are not fully understood. Growing evidence suggests that cancer cells are heterogeneous and are originated from cancer stem cells (CSCs), which are a minor population of cancer cells. In the present study, we investigated the effects of nicotine on the size of CSC population in human cancer cell lines. CSC population was analyzed by flow cytometry with a CSC functional marker aldehyde dehydrogenase. We examined whether nicotine increased a CSC population in human cancer cell lines, such as A549 (lung), MCF-7 (breast), PC-3 (prostate) and U-251 (glioma). We found that nicotine increase CSC population in MCF-7 cells, not in A549, PC3 and U251 cells. The nicotine-treated MCF-7 cells formed more mammospheres than untreated control cells, confirming that nicotine increases the CSC population. These data suggest that the effect of nicotine is selective in breast cancer. We next investigated the subtype of nicotinic acetylcholine receptor (nAChR). The effect of nicotine was blocked by the α7 subunit-selective antagonist of nAChR α-Bungarotoxin. In addition, the α7-selective nAChR agonist PHA543613 increased the CSC population in a dose-dependent manner. These data suggest that nicotine increases the CSC population via α7-nAChR. To investigate whether the effect of nicotine is mediated through a stem cell-dependent pathway, we examined the Notch pathway, which is a feature of CSC. We found that nicotine induced an expression of Notch target gene Hes1. DAPT, which inhibits cleavage of activated Notch receptors by γ-secretase and thereby prevents Notch signaling, reduced the nicotine-induced Hes1 expression and the nicotine-induced CSC population. Taken together, these data suggest that nicotine increases the CSC population via the α7-nAChR- and Notch-dependent pathway in MCF-7 cells. These findings reveal a relationship between nicotine and the CSCs in human breast cancer. This might explain the development of breast cancer in cigarette smokers.

Poster Board Number: T-2065

NOVEL GRP78 - TARGETING PEPTIDES FOR THERAPY AND IMAGING OF CANCER

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We had developed a strategy of structure-based optimization of cancer-targeting peptides to target human GRP78. Based on computer modeling, GRP78 composed of two major structural domains, a peptide-binding and an ATPase domain. GRP78 was reported to reside on the outer surface of cancer cells, but only in the cytoplasm of normal cells. Using our newly developed software, HotLig, for predicting protein-peptide interactions, a series of novel cancer-targeting peptides against GRP78 were designed *in silico* with molecular docking. The abilities of these peptides to bind to peptide-binding domain of GRP78 were further confirmed via Biacore analysis. We also demonstrated their capacities to target cancer cells through *in vitro* binding with a variety of cancer cells including clinical cancer specimens, and *in vivo* tumor imaging and targeted chemotherapeutic studies using peptide-linked liposome. Based on these analysis, we delineated a cancer-targeting motif Pro-X1-Leu-X2, where X1 could be amino acids with a hydrophobic side chain including Leu, His, Phe, and Tyr, and X2 could be Pro and aromatic amino acids Phe and Trp. Peptides possessing this motif, such as P-6, P-12 and P-13, target the peptide-binding domain of human GRP78, and could bind to a broad spectrum of cancer cells but not to normal cells. In addition, the novel peptide-linked Lipo-Dox displayed significant increases in therapeutic efficacy for human breast and lung cancer xenografts in mice as compared to the Lipo-Dox without targeting peptides. Besides, the microSPECT/CT imaging showed significantly greater uptake of radioactivity with ^{188}Re-peptide-linked liposome compared with ^{188}Re-liposomes alone, opening up a valuable application of these novel peptides for cancer diagnosis and targeted radiotherapy. Notably, these new cancer-targeting peptides possess the potential to target drug delivery to cancer initiating/stem cells, which might also provide a new paradigm for cancer stem cell-targeted therapy. In our recently established inducible Kras^{G12D}-driven lung adenocarcinoma model in *CCSP-rtTA/TetO-Cre/LSL-Kras^{G12D}* mice, we have identified bronchiolar Clara cells as the origin of cells which initiated Kras-induced tumor formation in the lung (Cancer Res.71 7250 2011). Now we also found that the expression of GRP78 in the tumorigenic EpCAM$^+$MHCII$^-$ subpopulation of cells in this *in vivo* lung cancer model was increased at least six folds, especially on the outer surface of cancer initiating cells. Similarly, the expression of GRP78 in the breast-cancer stem cell subpopulation in xenografts of human primary breast cancer was also significantly increased. Of interest, these peptides could bind to breast-cancer stem cell subpopulation

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in xenografts of primary breast cancer as well as $CD44^+CD24^-$ cells from clinical specimens of breast cancer. Therefore, these newly found and optimized cancer-targeting peptides were expected to enhance "tumor selectivity" for imaging and targeted therapy of cancers, including cancer initiating/stem cells, thereby increasing the therapeutic index of the anticancer drugs.

Poster Board Number: T-2066

ROLE OF RAT MESENCHYMAL STEM/STROMAL CELLS AS A CO-CONSPIRATOR FOR TUMOR PROGRESSION IN A RAT OSTEOSARCOMA MODEL:A COMPARATIVE ANALYSIS OF GENE EXPRESSION PROFILES IN RELATION TO TUMOR PROGRESSION

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INTRODUCTION: Mesenchymal stem/stromal cells (MSCs) are proposed as a source of tumor stromal cells during the progression of various cancers such as breast cancers. However, the role of MSCs still remains uncovered in most sarcomas, although the MSCs are considered to be the cell of origin for most of sarcomas. We will propose the role of MSCs in tumor growth and metastatic ability with comparative analysis of gene expression profiles based upon the results of preliminary experiments in rat osteosarcoma model. MATERIALS and METHODS: Two components of experiments have been performed using the cell lines of rat osteosarcoma COS1NR established from the tumor chemically induced by 4-hydroxy(amino) quinoline 1-oxide in F344 rats and rat MSCs isolated from syngeneic rat femur bone marrow. *In vivo* animal experiments were performed as the simultaneous co-implantation of both cells into subcutaneous tissues and the subcutaneous inoculation of osteosarcoma cells followed by subsequent intravenous injection of MSCs intermittently. Upon those experiments, we have analyzed the gene expression profiles using Agilent gene expression array comparing MSCs and osteosarcoma cells to identify the factors possibly involved in the process of tumor progression. RESULTS: The data of animal experiments indicated that MSCs could promote tumor engraftment and metastatic colonization in a rat osteosarcoma model. Briefly, simultaneous co-implantation of MSCs and osteosarcoma cells showed higher incidence of tumor formation and tumor growth rate in early phase compared to osteosarcoma cell inoculation alone. Intravenous MSCs injections after subcutaneous inoculation of osteosarcoma cells enhanced the formation of lung metastatic nodules in the group with MSCs injection compared to the group without MSCs, while no difference was observed in subcutaneous tumor growth between those groups. The pathway analysis from comparative gene expression profiles between syngeneic MSCs and osteosarcoma cells identified that pathways involved in cell cycle acceleration including CDKs-cyclins and DNA repairs were up-regulated in osteosarcoma cells, while genes involved in focal adhesion, cytokine-cytokine receptor, chemokine signaling and extracellular matrix-receptor pathways such as CAMs (ICAM and VCAM) - integrins including interleukins, CCL-CCRs, CXCL12-CXCR4, MMP-2 and 9, mostly related to tumor progression such as invasion and metastasis, were up-regulated in MSCs rather than osteosarcoma cells themselves, suggesting possible participation of MSCs in the tumor progression through the cell-cell and cell-matrix interactions as a co-conspirator. CONCLUSIONS: We hypothesize that MSCs could be a potential co-conspirator for osteosarcoma cells to enhance the ability of settlement and colonization leading to early onset of growth and metastasis. The activated pathways including cell adhesion, cytokines and chemokines, and MMPs in MSCs may be involved in cell-cell and cell-matrix interaction to promote tumor progression, potentially compensating the tumor cell ability and cultivating the microenvironments for tumor cells.

Poster Board Number: T-2068

CD138-NEGATIVE CLONOGENIC CELLS ARE PLASMA CELLS BUT NOT B CELLS IN SOME MULTIPLE MYELOMA PATIENTS

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Clonogenic multiple myeloma (MM) cells reportedly lacked expression of plasma cell marker CD138. It was also shown that CD19+ clonotypic B cells can serve as MM progenitor cells in some patients. However, it is unclear whether CD138-negative clonogenic MM plasma cells are identical to clonotypic CD19+ B cells. We found that in vitro MM colony-forming cells were enriched in CD138-CD19-CD38++, plasma cells, while CD19+ B cells never formed MM colonies in 16 samples examined in this study. We next used the SCID-rab model, which enables engraftment of human MM *in vivo*. CD138-CD19-CD38++ plasma cells engrafted in this model rapidly propagated MM in 3 out of 9 cases, while no engraftment of CD19+ B cells was detected. In 4 out of 9 cases, CD138+ plasma cells propagated MM, although more slowly than CD138- cells. Finally, we transplanted CD19+ B cells from 13 MM patients into NOD/SCID IL2Rγc-/- mice, but MM did not develop. These results suggest that at least in some MM patients CD138-negative clonogenic cells are plasma cells rather than B cells, and that MM plasma cells including CD138- and CD138+ cells have the potential to propagate MM clones *in vivo* in the absence of CD19+ B cells.

Poster Board Number: T-2069

A PUTATIVE CD34+ CANCER STEM CELL LINE WITH KUPFFER CELL CHARACTERISTICS PRODUCES A COMBINED HEPATOCELLULAR CHOLANGIOCARCINOMA

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CD34+ stem cells play an important role during liver development and regeneration. We hypothesized that human liver carcinomas (HLC) might be derived from oncogenically mutated or epigenetically aberrant CD34+ cells. We determined that a population of CD34+ stem cells isolated from PLC/PRF/5 hepatoma cells (PLC) and clonagenically expanded on mouse feeder layers appears to function as liver cancer stem cells (LCSC) by forming HLC xenografts in NOD/SCID/IL2rg mice after injecting only 500 CD34+ stem cells; whereas unsorted parental PLC required at least 100,000 cells to produce HLC. Thus, a small number of CD34+ stem cells had tumor initiation capacity. We then attempted to characterize the origin of these LCSC. 12 subpopulation of CD34+ stem cells (CD34+CD44±, CD34+CD133±, CD34+CD31±, CD34+CD90±, CD34+EpCAM±, and CD34+OV6±) were sorted and injected into NOD/SCID/IL2rg mice. HLC were formed in mice from all 12 subpopulations. By

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using flow cytometry, we found that the phenotype of the tumor cells produced by injecting CD34+ stem cells and its subpopulations were similar to tumors produced by injecting parental PLC. Hep Par1 was expressed in a high percentage of parental PLC cells; however, only a few cells were positive for CK19 and CD68. The tumor cells produced from the injection of parental PLC or from the CD34+ stem cells both co-expressed Hep Par1 and CK19 as a high percentage of population, showing the phenotype of combined hepatocellular cholangiocarcinoma (CHC). Interestingly, CD34+ double positive cells (except CD34+OV6+, and CD34+CD133+) expressed CK19 in a higher percentage of the populations when compared to those from the corresponding group for CD34+ negative for CD31, CD90, CD44, and EpCAM. Moreover, the tumor cells produced after the injection of either parental PLC or CD34+ stem cells expressed Kupffer cell (KC) markers: CD68, lysozyme, and CD14 in a very high percentage of the population, and did not express liver sinusoidal endothelial cell (LSEC) markers except CD31. Oval cells, LSEC and KC are all bone marrow-derived liver cells and express CD34; however, LSEC and KC express CD31, but oval cells do not. Moreover, oval cells are positive for OV6, whereas LSEC and KC are not. Thus, we were surprised by the finding that our putative LCSC were positive for OV6 and KC markers. In the adult liver, oval cells are thought to be an unhomogenous population of primary liver stem/progenitor cells, which are activated during progenitor-dependent regeneration. The isolated CD34+OV6+ population from parental PLC or cultured CD34+ stem cells also expressed CD68 as well as CD133, which is not expressed in normal liver cells, and did not express CD31, EpCAM, CD90, or CD44. Therefore, we conclude that the CD34+ stem cells that originated from CD34+OV6+ oval cells have KC characteristics, and function as LCSC with CD34+OV6+CD133+CD68+ stemness. The CD34+ double positive cells derived from these CD34+OV6+ cells show tumor initiation capacity and produce well-differentiated CHC. The tumor cells produced from injecting these CD34+-derived cells by serial transplantation co-expressed hepatocyte, cholangiocyte, and Kupffer cell markers as a homogenous population, and the xenografts yielded a well-differentiated CHC tumor with KC markers. This is the first report that a human CHC appears to be initiated and developed from CD34+ LCSC which have characteristics common to both oval cells and KC.

Poster Board Number: T-2070

PURIFICATION AND DEPLETION OF CANCER INITIATING CELLS CULTURED ON BIOMATERIALS HAVING NANOSEGMENTS

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Tumors contain a small subpopulation of cells, i.e., cancer stem cells (CSCs, cancer-initiating cells), which exhibit a self renewing capacity and are responsible for tumor generation. The cancer stem cells and not normal cancer cells persist in tumors as a distinct population, and cause relapse and metastasis by giving rise to new tumors. It is necessary to eliminate only a tiny subset of cells (0.0001-0.1%, cancer stem cells) that have the ability to generate a new tumour in cancer patients. If we succeed to develop biomaterials where CSCs are depleted or purified from tumor cells, it should be useful in clinical application. The purified CSCs can be used for the development of the specific anti-cancer drugs targeting only the cancer stem cells. We can save tumor patients with low side effects of medicine and avoid relapse and metastasis. On the other hand, the depletion of CSCs from tissue should be useful for the isolation of mesenchymal stem cells or bone marrow cells (hematopoietic stem cells) from patient tissue or blood. The mesenchymal stem cells or bone marrow depleting CSCs can be used for the stem cell therapy for the patients. Currently, surface markers and/or gene expression of CSCs are unknown, although CD34+, CD44+, CD133+, CD166+,Sca-1, Lgr5,and Muc2, etc are suggested. There are several contrary data suggesting those surface markers and/or genes are not specific to CSCs. The most promising method to quantify and identify CSCs is *in vivo* experiments where the sample cells are injected into mice subcutaneously, and to evaluate the tumor generation speed by the injection of the sample cells. In this study, several colon cancer cell lines (LoVo, Colo205, etc) and primary colon cancer cells from patients are cultured on tissue culture dishes (TCPS), extracellular matrix (ECM, collagen type I, fibronectin, vitronectin, orlaminin) coated dishes, and pluronic-grafted dishes. The pluronic is polyethylene oxide (PEO)-polypropylene oxide (PPO)-PEO triblock copolymers where it is reported that hematopoietic stem cells efficiently preserved on pluronic-grafted dishes. It is found thattumor generation of colon cancer cells was enhanced after the colon cancer cells were cultured on ECM-coated dishes, which indicates CSCs are enriched. On the other hand, tumor generation of colon cancer cells decreased after culture of colon cancer cells on pluronic-grafted dishes. Remarkably, tumor generation did not observed when primary colon cancer cells were cultured on pluronic-grafted dishes, which indicates CSCs in primary colon cancer cells are depleted after culture on pluronic-grafted dishes. The cells from fat cancer tissues were cultured on pluronic-grafted dishes, and adipose-derived stem cells (ADSCs) were isolated from the fat cancer tissues. These cells did not generate tumor on mice, while the cells can differentiate into osteoblasts, which indicates ADSCs are safely isolated without contamination of CSCs after culture on the pluronic-grafted dishes. On the other hand, ADSCs isolated from fat cancer tissues, which cultured on conventional TCPS or ECM-coated dishes generated tumor on mice, which indicates ADSCs have contamination of CSCs when the fat cancer cells are cultured on TCPS or ECM-grafted dishes. It is concluded that the pluronic-grafted surface deplete cancer-initiating cells (CSCs) from colon cancer cell lines and primary cancer cells, while CSCs in colon cancer cells are enhanced by culture on conventional TCPS and ECM-grafted dishes promote CSCs.

Poster Board Number: T-2071

PROLIFERATION OF HUMAN PLURIPOTENT EMBRYONAL CARCINOMA STEM CELLS IS INHIBITED BY CO-CULTURE WITH THEIR DIFFERENTIATED PROGENY

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The 'cancer stem cell theory' proposes that undifferentiated tumour-initiating cells behave akin to stem cells to both self-renew and produce the differentiated cells that form the bulk of the tumour. A high proportion of undifferentiated cells is the main determinant in defining a high grade tumour with poor prognosis. In vivo, undifferentiated and differentiated tumour cell types reside in the same environment. Undifferentiated and differentiated cancer cells have been isolated from several tumour types and comparatively analysed in isolation. However, our understanding of how these different populations may interact in vivo is poor. This was addressed through PASCA (www.pasca.eu), a European Union 7th Framework funded project aiming to characterise the heterogeneous nature of cancers. NTera2 human pluripotent embryonal carcinoma stem cells were retinoic acid-differentiated for one week. These cells were then co-cultured with undifferentiated NTera2 cells at ratios of 1:1 or 9:1 to broadly model the in vivo tumour.

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After co-incubation, undifferentiated and differentiated cells were separated via flow cytometry based on the expression of Stage Specific Embryonic Antigen 4 (SSEA4). Proliferating undifferentiated (SSEA4+) cells were expected to outgrow terminally differentiated (SSEA4-) cells unless inhibited by the co-culture. Differentiated and undifferentiated cells mixed at 1:1 and 9:1 ratios could be immediately correctly discriminated by flow cytometry to approximately 1:1 or 9:1 proportions based on SSEA4 expression. In 1:1 co-cultures, SSEA4+ cells outgrew SSEA4- cells over time as expected. However, two weeks of 9:1 co-culture was sufficient to substantially slow this proliferation of SSEA4+ cells. Co-culture with two-week differentiated cells amplified this inhibition, which now took place earlier. Next, the requirement for cell-cell contact in this inhibition was assessed. SSEA4+ cells were incubated with conditioned media from either SSEA4+ or SSEA4- cells. SSEA4+ conditioned media had little effect on the cells or their SSEA4 flow cytometry profile. However, after one week, cells treated with SSEA4- conditioned media had reduced proliferation and increased size compared to cells treated with SSEA4+ conditioned media. Additionally, incubation with SSEA4- cell conditioned media shifted the SSEA4 profile between that of undifferentiated and differentiated cells. Collectively, this data indicates that differentiated cells communicate with and regulate the growth of their parental undifferentiated cell in a cancer stem cell model of tumour biology. Regulation requires an excess of differentiated cells and is amplified as differentiated cells mature. This effect is partially due to products secreted by differentiated cells but requires the presence of differentiated cells for maximum effect. Mechanistic analysis continues and will be reported upon. The intriguing question of whether embryonic stem cells are regulated by the differentiated cells in their in vivo environment remains.

Poster Board Number: T-2072

P53 DEFICIENCY IN MESENCHYMAL STEM CELLS CONTRIBUTES TO TUMOR DEVELOPMENT BY SHIFTING IMMUNE MICROENVIRONMENT

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Tumor development is often associated with mutations in tumor suppressor genes. Most studies focus on the alterations by these mutations in tumor cells, while the changes in stromal cells are rarely investigated. Mesenchymal stem cells (MSCs) are continuously attracted to tumors and become a key component of the tumor microenvironment. Recent studies have demonstrated that MSCs play a critical role in regulating immune responses. However, whether mutations in tumor suppressor genes in MSCs could affect tumor progression is not known. We isolated MSCs from wild type and p53 deficient mice and investigated their effect on tumor growth. We found that wild type MSCs had no effect on tumor growth. MSCs with the deficiency in p53 significantly enhanced tumor growth in mice with normal immune system, but not in NOD/SCID mice. Moreover, neither p53 deficient MSCs nor wild type MSCs affected the proliferation of tumor cells, indicating that the tumor promoting effect on $p53^{-/-}$ MSCs is exerted through immune reactions. Furthermore, p53 deficiency in MSCs leads to elevated *iNOS* expression and nitric oxide production when cells were stimulated with inflammatory cytokines interferon-γ and tumor necrosis factor-α. This increase in NO production leads to more prominent immunosuppression, which could be abolished by *iNOS* inhibitor L-NMMA. Taken together, p53 deficiency in MSCs promotes tumor growth through inhibiting immune response. Modulation of p53 expression in stromal cells is a novel target for cancer therapy.

Poster Board Number: T-2073

A NOVEL PRO-REPROGRAMMING FACTOR, GLIS1, IS REGULATED BY HYPOXIA-INDUCIBLE FACTOR

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GLI-similar 1 (GLIS1) is a member of Krüppel-like zinc finger transcription factors that are closely related to GLI family. During mouse embryonic development, *Glis1* was reported to be expressed in a temporal and spatial manner; expression was prominent in several defined structures of mosodermal lineage, including craniofacial region, branchial arches, somites, vibrissal and hair follicles, limb buds, and myotomes. In adult mice, *Glis1* expression was observed abundant in placenta and adult kidney, and low in testis, thymus, colon, brain, and adipose tissue. Furthermore, significant induction of *Glis1* expression was observed in psoriatic epidermis and in mouse skin with the tumor promoter phorbor-12-myristate-13-acetate (PMA) treatment. However, the molecular mechanisms of regulation of *Glis1* expression in both physiological and pathological conditions are mostly undetermined. Very recently, Glis1 was demonstrated to enhance markedly the generation of induced pluripotent stem cells (iPSCs) from both mouse and human fibroblasts when it is expressed together with OCT3/4 (POU5F1), SOX2, and KLF4. DNA microarray analyses revealed that Glis1 effectively promoted the direct reprogramming of somatic cells during iPSCs generation through activation of multiple pro-reprogramming pathways. On the other hand, it was demonstrated that iPSCs generation was also enhanced in hypoxic condition, although underlying molecular mechanisms was still unclear. We thus examineded whether hypoxia regulate GLIS1 expression, and tried to clarify behind the molecular mechanisms behind this. At first, variety of cell lines - MCF-7, MDA-MB-231, SK-BR-3 breast cancer cells; HSC-2, HSC-3, HSC-4, Ca9-22, KOSC-2 oral cancer cells; KD lip fibroblasts; A549 lung cancer cells; HepG2 liver cancer cells; RCC4/pcDNA, RCC4/VHL kidney cancer cells; SK-N-BE(2)-C neuroblastoma cells; HCT116 colorectal cancer cells - were incubated under normoxia (21% pO_2) or hypoxic conditions (1% pO_2) for 24 hours. Real-time RT-PCR analyses demonstrated that *GLIS1* expressed relatively higher in MCF-7 and RCC4, and at low levels in other cells tested. Interestingly, *GLIS1* expressions were dramatically increased in hypoxic conditions in many cell lines as well as known hypoxia-induced genes, *ADM* and *CA9* and pro-reprogramming genes, *POU5F1* and *KLF4*. Time course experiments showed that expression of *GLIS1* was gradually increased until 48 hours in hypoxic MCF-7 cells. Renal cell carcinoma cell line RCC4 is known as a cell line with inactivated tumor suppressor gene, von Hippel-Lindau (VHL), resulting constitutive activation of HIF-signaling pathway. Comparison between parental RCC4/pcDNA and revertant RCC4/VHL showed that pVHL down-regulated *GLIS1* expression. Furthermore, knock-down experiments demonstrated that inhibition of HIF-1 abolished hypoxic induction of *GLIS1* expressions. These results suggest that the VHL-HIF-1 pathway may play a pivotal role in the generation of iPSCs and/or maintenance of tumor-initiating cells through promoting expression of pro-reprogramming factor genes, such as *GLIS1*. Further analyses are now on-going and details of mechanisms of GLIS1 regulation and function will be presented.

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Poster Board Number: T-2074

REGULATION OF CANCER STEM CELL ACTIVITIES BY IMMUNOLOGICAL NICHE

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Research background: Tumor microenvironments play a critical role in regulation of activities and survival of cancer stem cells (CSC) that are responsible for rendering tumors to resist various anticancer modalities. Recent studies have been unveiled that indicate tumor-associated macrophages (TAM) regulate tumor progression and metastasis, but it remains unclear whether interplay between cancer stem cells and immunological niche regulate CSC activities. Therefore, we evaluated the contribution of TAM in the regulation of tumorigenic activities and anticancer drug sensitivities of CSC. Experimental results: We used several soluble/cell surface proteins, such as MFG-E8, IL-6, ariginase-I, CCL-2, IDO,TIM-3,TIM-4 as functional markers produced from TAM because they are highly produced from TAM and play a critical role in triggering anticancer drug resistance and tumorigenesis. We verified in this study that CSC, but not other tumor subsets, could upregulate TAM-derived tumorigenic factors (referred as TAM-TF) from normal macrophages. Furthermore, *in vivo* adoptive cell transfer model revealed that TAM-TF are responsible for maintaining tumorigenic activities of CSC, such as self-renewal capacities and anticancer drug resistance. The pharmacological inhibition of several TAM-TF with neutralizing antibodies significantly reduced tumor burden and increased anticancer drug sensitivities of primary human and murine CSC in preclinical models. Through screening oncogenic signals responsible for TAM-mediated activation of cancer stem cells, we found that Stat3 and Hedgehog pathways are responsible for enriching and activating cancer stem cells in bulk tumor populations. Significance: We identified positive feedback mechanisms whereby CSC is responsible for converting normal macrophages into those with tumor-promoting activities. In turn, CSC-modified macrophages produced several tumorigenic factors that are critical to activate self-renewal and tumorigenic activities of CSC. The molecular targeting of these positive-feedback pathways provides the new therapeutic strategy to eradicate treatment-difficult tumors across the broad spectrums of different genetic and epigenetic alterations.

Germline Cells

Poster Board Number: T-2075

MIR-372/LET-7 AXIS IN HUMAN PRIMORDIAL GERM CELL SPECIFICATION

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The embryonic stem cell-specific cell cycle-regulating (ESCC) microRNA (miRNA) family including miR-372 promotes while the let-7 family inhibits pluripotency. Here we show that during differentiation of human embryonic stem cells (hESC), somatic lineages activate let-7 and silence the ESCC miRNA family. In contrast, induced primordial germ cells (iPGC) keep let-7 silenced while retaining expression of miR-372. Introduction of let7 into differentiating hESC block iPGC specification, in part due to its ability of to suppress Prdm1. In contrast, miR-372 promotes differentiation of hESC into iPGCs by suppressing targets in multiple cellular processes including cell cycle, epithelial-mesencymal transition (EMT), and epigenetic regulation. These same targets were shown previously to promote reprogramming of human fibroblasts to induced pluripotent stem cells (iPS) uncovering common pathways of miRNA regulation in these two transitions. These findings identify a novel role for the ESCC and let-7 miRNAs in regulating cell fate choice between somatic and germline lineages.

Poster Board Number: T-2076

DERIVATION OF POST MEIOTIC GERM CELLS FROM HUMAN EMBRYONIC STEM CELLS

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Investigating the mechanisms of human primordial germ cell (PGC) and gamete development is important for understanding the causes of infertility, effects of chemicals on reproductive development and cancer; and may eventually lead to new clinical applications. The aim of this study was to investigate a monolayer method to isolate PGCs and later germ cells. Shef hESC lines (Shef 2 and 4) were cultured on mouse embryonic feeders (MEFs) and allowed to undergo spontaneous differentiation in medium supplemented with retinoic acid. Differentiation to germ cells was monitored using cell surface markers and mRNA expression by Q-PCR. There are six novels gene meiosis marker being introduced to determine the differentiated primordial germ cells using Q-PCR. Fluorescent in-situ hybridization (FISH) has been done to detect the cell undergo meiosis with random probe. After 4-7 days in culture with retinoic acid, up to 20% of hESCs displayed a PGC phenotype as determined by antibody markers (ckit, SSEA-1, VASA, DAZL) although there was batch to batch variation. After 7 days of culture, there showed an increase of genes meiosis marker expression in culture supplemented with retinoic acid. The expression of germ cell-specific mRNA correlated with proportion of cells exhibiting appropriate surface marker. We manage to get a small population of cells undergo meiosis using FISH. The cells were then re-cultured using neonatal mouse testis conditioned media (NMTCM) for 45 days. After Q-PCR analyses and staining with specific post-meiotic markers, we managed to identify the positive population of post-meiotic cells. The culture have been continued using NMTCM added with spermatogenesis hormones: Follicle Stimulating Hormone, Luteinizing Hormone, and Testosterone for 15 days. The development of post meiotic cells were increased significantly using this approach.

Poster Board Number: T-2077

EVIDENCE OF PRIMORDIAL GERM CELL IN HUMAN OVARIAN ENDOMETRIOTIC LESIONS

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Endometriosis is a common gynecological disorder affecting 10% of all women. This pathology is characterized by dysmenorrhea, dyspaneuria, pelvic pain, and infertility. In some cases, malignant transformation occurs in the lesion. Although this disorder was described in 1860, it still remains an enigmatic disease, mainly in its etiology. Sampson´s implantation theory is the most accepted explanation on the origin of endometriosis proposing that endometrial cells exfoliated during menstruation reflux through the uterine tubes, adhere to and proliferate at ectopic sites. Recently, the presence of stem cells in ovarian endometriosis was demonstrated; these cells could be involved in the progression of the disease and its malignant transformation. We propose that ovarian stem cells,

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lying in the gonad to renew the germinal mass, initially recruited to develop into primordial germ cells (PGC), act afterwards in the disease progression as a consequence of the hormonal environment of the endometriotic lesion. The main objective of this work was to search for the existence of PGC at ovarian endometrioma. Five samples were collected from patients, after obtaining their informed consent, according to the following criteria: histopathological diagnosis of ovarian endometriosis, women aged 18 to 35 years old, normal menstrual cycle and no history of any hormonal therapy. As control samples we used endometrial tissue both in proliferative and secretory phase (n=2 for each). Samples were processed by immunohistochemistry and immunofluorescence for: DDX4, IFITM1, IFITM3, OCT4, SSEA4, Progesterone receptor (PGR), Estrogen receptor alpha (ESR1), PCNA and CD45. Germ cell markers IFITM3 and DDX4 were strongly immunostained in clock face-arranged cells with central nucleolus. These cells also displayed nuclear signal for ESR1, PCNA and SSEA4 but were negative for PGR, OCT4 and CD45. Positive cells for PGR, ERS1, OCT4 and PCNA were found in the stroma of all the endometriotic lesions and endometrial control samples. The expression of PGR, DDX4, IFITM1 and IFITM3 was analyzed by RT-PCR from formalin-fixed paraffin embedded tissue. DDX4, IFITM1, and IFITM3 mRNAs were expressed in all endometriotic samples but not in endometrial tissues. The presence of DDX4, IFITM1 and IFITM3 indicates the existence of PGC in ovarian endometriotic lesions. Additionally, the expression of ESR1 suggests that these cells were probably recruited due to estrogenic stimulation from ovarian stem cells, with loss of OCT4 activity while maintaining the expression of SSEA4. These results outline ovarian endometriosis as a model for studying the renewal of the germinal mass in the female gonad.

Poster Board Number: T-2078

FUNCTIONAL ANALYSIS OF PIWIL1 IN COMMON MARMOSET

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Piwi-interacting RNAs (piRNAs) are a distinct class of germline-specific small non-coding RNAs. piRNAs associate with germ line-specific Argonaute proteins, or PIWI proteins, to form piRNA-induced silencing complexes (piRISCs) and function in transposon silencing. In mice, MIWI (PIWIL1), MILI (PIWIL2) and MIWI2 (PIWIL4) are classified as PIWI proteins. Mutations in the Piwi genes cause de-repression of transposons in testes and male sterility. Interestingly, however, each PIWI protein shows distinct expression pattern through development. MILI expression can be observed starting from primordial germ cells to round spermatids. MIWI expression is found in pathytene spermatocytes to round spermatids. MIWI2 are only found from E12.5 to 3 days after birth in G1-phase arrested gonocytes. In embryonic testis, MILI and MIWI2 are localized in the pi-bodies and piP-bodies, respectively, both of which are known to be nuage, germ line-specific, high electron dense granules appearing around the nucleus. On the other hand, in adult testis, MIWI and MILI co-localize in pi-bodies. In addition, each PIWI associates with different piRNA populations. Evidence has shown that the piRNA pathway associates with DNA remethylation in the transposable elements and also in the paternal imprinted loci in the gonocytes, like Dnmt3l mutants. Thus, the PIWI-piRNA pathway in mice is essential for spermatogenesis. Unlike rodents, primates including humans encode four members of the PIWI genes (through PIWIL1 to PIWIL4). This may suggest that the PIWI-piRNA-mediated silencing mechanism in primates differs from that in rodents. However, the PIWI-piRNA pathway remains to be elucidated. Here, we study the primate PIWI-piRNA pathway in common marmoset, Callithrix jacchus. We produced a monoclonal antibody against marmoset PIWIL1 (an ortholog of MIWI), which we named as MARWI. Immunofluorescence analysis showed that MARWI is expressed in spermatocytes to round spermatids in the juvenile and adult testis, but not in the neonatal testis. Furthermore, immunoelectron microscopic (iEM) analysis revealed that MARWI is localized nuage-like, high electron dense granules in the cytoplasm. Immunopurified MARWI associates with small RNAs of ~30 nucleotides. Currently, we are engaged in determining sequences of MARWI-associated piRNAs. We will report characterization of MARWI-associated piRNAs.

Poster Board Number: T-2079

DERIVATION OF PRIMORDIAL GERM CELLS FROM MOUSE EMBRYONIC STEM CELLS IN A GROUND STATE PLURIPOTENCY.

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Recently, successful derivation of germ cells which showing capacity of fertilization has been reported. However the successful rates of germ cells are not uniform between each study. In order to apply the technology of *in vitro* germ cell production for research of infertility or clinical treatments, development of the method with high reproducibility is essential.Now, it has been demonstrated that the mouse embryonic stem cells (mESCs) show heterogeneous populations, in which the gene expressions could be varied in each individual cell. This characteristic of the mESCs could lead to susceptibility by culture conditions in undifferentiated status, and it also could result in low reproducibility when the cells are induced differentiation into germ cell lineage. In this study, we first produced a mES cell line containing Oct4deltaPE-GFP construct, which enables monitoring primordial germ cell derivation by GFP expression, cultured it in the condition administrated with a GSK3b specific inhibitor CHIR99021 and a MEK specific inhibitor PD0325901 (referred as 2i), which forcibly stabilized the mESC pluripotency in a ground state (reffered as 2i-ESCs), and then efficiency of the germ cell differentiation was evaluated following spontaneous differentiation. When observed at day-9 from differentiation induction, increasing GFP-positive fractions that expressed Mvh and Stella were observed in the derivative cells from the 2i-ESCs. However, in the derivatives from control ESCs without 2i treatment at this time-point, rates of the GFP-positive fraction were significantly varied, and marker gene expressions in it were significantly lower than that of the derivatives of the 2i-ESCs. When transplanted the day-9-derivative cells from the 2i-ESCs into seminiferous tube of B6WB-W/Wv mice, successful engraftment and contribution into the region of a spermatogonia-stage cells could be observed. These results suggested that the preliminary treatment of the ESCs in the 2i-added condition to induce ground state pluripotency could improve the efficiency of the method for germ cell differentiation induction.

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Poster Board Number: T-2080

MIRNA REGULATION ON THE UNDIFFERENTIATED STATE OF CHICKEN PRIMORDIAL GERM CELLS

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In early germ cell development, primordial germ cells (PGCs) are should be regulated by transcriptional and post-transcriptional level with tight spatial and temporal control for the unique characteristics distinguishing themselves from other somatic cells. MicroRNAs (miRNAs) are small non-coding RNAs, being between 18~25-nucleotides length, that post-transcriptionally regulate transcripts and function by binding with their target mRNA through base-pairing with the 3'untranslated region (3'UTR). Moreover, in mouse, it has been known that miRNA biogenesis is critical for the PGCs development. However, the complex networks of developmental processes especially in miRNA are less understood in chicken PGCs. Thus, we identified specifically expressed miRNAs at the post-transcriptional levels in chicken PGCs and investigated a PGC-specific miRNA and its function. Here, we generated microarray analysis for miRNA expression between 6-day embryonic primordial germ cells and gonadal somatic cells (GSCs) sorted by FACS. Based on microarray, we identified ten miRNAs that are highly expressed in PGCs. Among them, miR-181a* showed the bi-function by negative regulation on two different transcripts. Interestingly, by repression *HOXA1* gene, miR-181a* prevented the somatic differentiation of PGCs. Also, miR-181a* inhibited PGCs from entering meiosis by down regulation of *NR6A1*. Our discoveries demonstrate that miRNA contribute to modulating differentiation and maintaining of undifferentiated properties in PGCs.

Poster Board Number: T-2081

LONG-GLUCOCORTICOID-INDUCED LEUCINE ZIPPER INTERACTS WITH RAS PATHWAY AND IS ESSENTIAL FOR SPERMATOGENESIS

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In the tightly controlled process of spermatogenesis undifferentiated self-renewing spermatogonia give rise to mature spermatozoa throughout life. The balance between undifferentiated spermatogonia self-renewal and differentiation maintains normal spermatogenesis and fertility, but cellular pathways regulating undifferentiated spermatogonia proliferation, differentiation and survival are only partially known. Here we show that long-GILZ (L-GILZ) is highly expressed in spermatogonia and primary spermatocytes, and is essential for spermatogenesis. Within first cycles of spermatogenesis, gilz deficiency in knock out (gilz KO) mice leads to a complete loss of germ cell lineage, resulting in male sterility. Spermatogenesis failure is not due to defects in endocrine or stem cell niche compartments but is intrinsic to germ cells and is associated with increased proliferation and aberrant differentiation of undifferentiated spermatogonia, and with hyper-activity of Ras signaling pathway as indicated by increase of Erk and Akt phosphorylation. Spermatogonia differentiation does not proceed beyond the prophase of the first meiotic division due to massive apoptosis associated with accumulation of unrepaired chromosomal damage. These results identify L-GILZ as an essential regulator of undifferentiated spermatogonia survival and germ lineage maintenance.

Poster Board Number: T-2082

IN VITRO PROPAGATION OF PRESUMPTIVE GERM CELLS WITH PRIMORDIAL GERM CELL CHRACTERISTICS DERIVED FROM MOUSE INDUCED PLURIPOTENT STEM CELLS

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The successful cultivation of germline stem cells (GSCs) from testis has opened up the possibility of the molecular analyses of mammalian spermatogonial stem cells. In contrast, primordial germ cells (PGCs) transform to the pluripotent state in culture, which has prevented a detailed investigation of their properties. This study demonstrates the *in vitro* expansion of presumptive PGCs derived from mouse induced pluripotent stem cells (iPSCs), without the reacquisition of pluripotency and tumorigenicity. The cells expressed both migratory and postmigratory PGC marker genes, and retained the parental genomic imprint. Interestingly, the imprint was erased in the neonatal testicular environment, suggesting that the developmental potential of the iPSC-derivatives reflected that of PGCs which had colonized the genital ridge. The induction and propagation of cells with PGC characteristics should serve as a conventional strategy for understanding mammalian germ cell development in the fetal gonad.

Poster Board Number: T-2083

DAZL IS A KEY REGULATOR OF PLURIPOTENCY IN THE GERMLINE

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Embryonic germ cell lines (EGs) derived from primordial germ cells (PGCs) are known to exhibit hallmarks of pluripotency, including teratoma and chimaera formation. This potential is maintained from E8.5 up to E12.5 in mouse development, at which the PGCs begin to undergo sexual differentiation. Dazl orthologues are essential for oogenesis and spermatogenesis across vertebrate species, and in mice, Dazl was found to be important in multiple steps of germ cell development, although the underlying molecular mechanisms are still unclear. Dazl belongs to the DAZ protein family, which is comprised of three mammalian members: DAZ (Deleted in Azoospermia), Dazl and Boll (*Drosophila* boule-like). Each of these members contains an N-terminal domain with RRM motifs, which presumably interact with RNAs, and a C-terminal domain containing one or multiple DAZ motifs with unknown molecular function. Previous biochemical studies of Dazl were restricted to

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adult testicular lysates, as the inaccessibility and paucity of the primordial germ cells (PGCs) and gonocytes present a major hurdle to our understanding of early germ cell development. We have developed employed an in vitro germ cell differentiation system to study the protein-RNA and protein-protein interactions of Dazl in early PGC development. We have identified mRNA transcripts that interact directly with Dazl and have found that Dazl specifically regulates the expression of core pluripotency factors, during in vitro as well as in vivo PGC development. We hypothesise that Dazl functions as a molecular switch, limiting germline pluripotency and thus allowing the initiation of meiosis.

Poster Board Number: T-2084

ELUCIDATION OF THE PRIMARY PIRNA PATHWAY IN *DROSOPHILA* OVARIAN SOMATIC CELLS

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PIWI proteins, germline-specific members of Argonaute family, function in RNA silencing in the gonad by associating specifically with PIWI-interacting RNAs (piRNAs). piRNAs arise mainly from intergenic repetitive elements including transposable elements (TEs) and their remnants. The PIWI-piRNA complex therefore is able to silence parental TEs, and also other genes when they show complementarities to the piRNA sequences. Without the functions of PIWI proteins and piRNAs, TEs become active, which has great potential to injure the genome; thus, both PIWI proteins and piRNAs are essential for maintenance of the genomic integrity and germline development. piRNAs in *Drosophila* ovarian somatic cells (OSCs) are generated only through the primary processing pathway and are loaded onto Piwi, a member of PIWI proteins. We have previously shown that primary piRNA biogenesis occurs through cytoplasmic granules called Yb bodies in OSCs. Both Armitage (Armi) and fs(1)Yb (Yb) proteins, core components of Yb bodies, are required for piRNA biogenesis. Armi guides nascent Piwi to Yb bodies by bridging the two proteins. This association leads the Armi-Piwi complex to be localized to Yb bodies. Recently, we found that Armi and Yb individually associate with piRNA intermediates and that Yb is required for assembly of the Armi-piRNA intermediate complex, further indicating the functional relevance of Armi/Yb in the piRNA biogenesis. We also found that Zucchini (Zuc), a member of the phospholipase D family, is required for primary piRNA biogenesis. Zuc localizes to mitochondria and plays an indispensable role in Yb body formation. To further understand the molecular functions of Yb bodies, we performed mutation analysis of Yb, a putative RNA helicase. A point mutation introduced in the Yb helicase ATP-binding site affected Yb body formation and primary piRNA production, suggesting that the interaction between Yb and putative piRNA precursors is a crucial step in the primary piRNA pathway. We also performed RNA fluorescence in situ hybridization (RNA FISH) to determine the intracellular localization of piRNA precursors. A piRNA cluster, flamenco (flam), gives rise to abundant piRNAs in OSCs. An RNA FISH probe recognizing the flam transcript detected dot-like flam signals in the cytoplasm. Double staining with anti-Yb antibodies showed that the flam signals were often localized adjacent to Yb bodies. These results suggest that primary piRNA precursors (or intermediates) are stored near Yb bodies until piRNA maturation finally occurs in Yb bodies. We are currently investigating how the flam signals in OSCs are affected by depletion of Armi, Piwi, Zuc and Yb.

Poster Board Number: T-2085

SETTING THE STAGE TO USE *DE NOVO* MORPHOGENESIS OF PORCINE TESTES TISSUE FOR GERM LINEAGE STEM CELL DIFFERENTIATION.

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Pluripotent stem cells can differentiate early germ cells in vitro, providing a model to study germ lineage differentiation and a potential avenue to generate gametes from infertile donors. However, differentiation beyond early germ cell stages is challenging and requires the three-dimensional testicular environment. Isolated testicular somatic and germ cells are able to recapitulate testicular morphogenesis and form a functional germ line stem cell niche upon transplantation into an immunodeficient mouse; however, currently the efficiency of *de novo* formed tissue supporting spermatogenesis is low and needs to be improved to allow study of the stem cell niche, testis development and to support spermatogenesis. This study tested the hypotheses that enrichment of spermatogonia prior to grafting or the presence of a scaffold would improve the formation of seminiferous tubules supporting spermatogenesis. Methods: Cells were harvested from the testes of 1 week old piglets by two step enzymatic digestion followed by a 3-step differential adhesion culture over 72 hours to attain germ cell enrichment. Cell recovery and viability were recorded and immunocytochemistry for UCH-L1 and vimentin was used to identify germ cells (UCH-L1 +, vimentin -) and somatic cells (UCH-L1 -, vimentin +). The enriched germ cell population (48.7±4.2%) was combined with the initial cell suspension in six different groups and grafted under the dorsal skin of castrated SCID mice (6 mice with 1 pellet per treatment group). Group 1: 50x106 cells containing 5% spermatogonia, Group 2: 50x106 cells containing 25% spermatogonia; Group 3: 50x106 cells containing 5% spermatogonia in Matrigel with reduced growth factorstm (MRGF); Group 4: 10x106 cells containing 5% spermatogonia in MRGF; Group 5: 10x106 cells containing 25% spermatogonia in MRGF; Group 6: 10x106 cells containing 25% spermatogonia. The degree of reconstitution of spermatogenic tissue was assessed by immunohistochemical localization of *de novo* formed tubules and germ cells (UCHL-1+) in all cross sections of tubular structures formed 24 weeks after transplantation. Results: GSC enrichment and the presence of the scaffold providing anchorage to the cells contributed to increased tubule formation and tubules supporting germ cell development when compared to Group 1 (Control group). Total number of *de novo* formed tubules and percentage of tubules supporting spermatogenesis per group was Group 1: 53.6±63.0, 20.8±9.9%; Group 2: 193.5±245.8, 35.1±24.3%; Group 3: 370.0±468.8, 36.4±13.8; Group 4: 122.6±23.8, 36.6±8.6%; Group 5: 89.3±92.2, 18.3±21.1%; Group 6: 61.3±40.6, 33.5±20.3, respectively. Despite the large variability encountered in the recovered newly formed tissue from all groups, the overall results supported the hypotheses. Therefore, enrichment of germ cells and presence of an extracellular matrix scaffold improve the efficiency of this bioassay. Given the high number of cells required to attain enrichment of the initial cell population the use of treatment 2 may be impractical, but our results indicate that the presence of MRGF can overcome germ cell loss and promote sufficient support to improve tubule formation. Consequently, this approach will provide an efficient bioassay system to investigate germ cell differentiation from germ line stem cells as well as from pluripotent cells.

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Poster Board Number: T-2086

HISTONE H3K27 DEMETHYLASE JMJD3 REGULATES THE TURNOVER OF SPERMATOGONIAL STEM CELLS.

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During spermatogenesis, epigenetic modifications of the genome, such as covalent modifications of histone residues, regulate chromatin structure and transcription and ensure appropriate gene activation and repression. Recent studies have provided evidence that modifiers of histone methylation are important for self-renewal and/or differentiation of stem cells. The renewable population of cells for spermatogenesis are the spermatogonial stem cells (SSCs). However, the roles of histone demethylases in spermatogenesis as well as spermatogonial stem cell renewal and differentiation remain elusive. We have discovered that histone H3 lysine 27 (H3K27) specific histone demethylase, JMJD3 (also known as KDM6B), is expressed higher in spermatogonial stem cells in culture than in neonate and adult testis. Expression of Jmjd3 in spermatogonial stem cells is decreased by removal of glial cell line derived neutrophic factor (GDNF), an essential growth factor for self-renewal of spermatogonial stem cells. Immunofluorecsent staining showed that JMJD3 localizes to PLZF-positive spermatogonia. Tri-methylated and di-methylated histone H3K27 that are methylation targets of JMJD3 formed punctate foci in PLZF-positive spermatogonia. In addition, H3K27me3 increased in differentiating spermatogonial stem cells in culture. When Jmjd3 levels were knocked-down in spermatogonial stem cells, spermatogonial stem cells, surprisingly, formed more colonies than control. Most colonies formed single, 2- and 4-aligned colonies when JMJD3 was knocked down, whereas there are many 8- 16 aligned colonies in the control culture. We also found some cells were not connected with neighboring cells, even if they were in same colony. To address the *in vivo* role of JMJD3 in spermatogonial stem cells, JMJD3 conditional knockout mice were generated. Unexpectedly, germ cell specific JMJD3 knockout mice were fertile and produced pups even at one year after mating. When spermatogonia in seminiferous tubule were visualized by PLZF staining, there were more single spermatogonia in JMJD3 deficient tubules than in control tubules. Cell surface marker analyses revealed that undifferentiated spermatogonia were increased in JMJD3 null testes. Molecular analyses showed that some genes expressed in undifferentiated spermatogonia as well as some genes expressed in differentiating spermatogonia were increased in the spermatogonia-enriched fraction from JMJD3-deficient testes. These results suggest that JMJD3 could regulate turnover of spermatogonial stem cells by repression of dedifferentiation of spermatogonial stem cells. We believe that our results provide further information on epigenetic regulation of stem cells as well as unique regulation of spermatogonial stem cells.

Poster Board Number: T-2087

NICHE-DEPENDENT REGULATION OF GERM CELL DEVELOPMENT

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The transmission of genetic information from one generation to the next depends on the production of functional male and female gametes, the sperm and egg, respectively. Primordial germ cells (PGCs) are the embryonic precursors of gametes and during development, interact with a continuously changing microenvironment or "niche". The ability to generate PGCs and subsequently differentiate them to functional gametes in vitro has been challenging, and identification of niche signals that regulate key maturation steps in PGC development may facilitate germ differentiation in vitro. Previous attempts to differentiate embryonic stem cells (ESCs) into germ cells suggest that development in embryoid body culture reaches the equivalent of a migratory e9.5 PGC by gene expression and epigenetic profiling. Using ESCs carrying the Oct4(ΔPE)-GFP transgene, we similarly obtained a PGC-like (esPGC) population that was highly enriched for transcripts of PGC-specific genes Stella, Blimp1, and Nanos3, but late PGC genes Dazl, and Mvh were low at the transcript level and absent by immunostaining in this population. Interestingly, Dazl and Mvh upregulation in esPGCs did not result even after prolonged embryoid body culture, suggesting that this microenvironment cannot support the completion of PGC differentiation. In an attempt to drive further PGC differentiation, we co-cultured esPGCs with somatic gonad & mesenephros cells (SGM) from embryos. After 3 days in co-culture, esPGCs up-regulated both DAZL and MVH, indicating that differentiation of esPGCs to mature PGCs is possible and dependent on the presence of SGM cells. *In vivo*, migratory PGCs arrive at the developing SGM beginning ~e10.5, which is followed by the upregulation of DAZL and MVH. To determine whether expression of these PGC maturity markers requires interaction with developing gonads, we cultured PGCs isolated from e9.75 and 10.5 embryos in the absence of somatic cells. After 12 or 24 h of culture, DAZL immunostaining could be detected in 54% of PGCs isolated at e10.5, but only 21% of e9.75 PGCs, suggesting that the SGM environment confers PGC competence or directly induces expression of mature markers *in vivo*. Through analysis of gene expression in PGCs and SGM, we have identified candidates for these required maturation cues from the SGM microenvironment, and are screening these candidates in embryo-derived as well as esPGCs. Together, the results indicate an important role for supporting niche cells in regulating PGC development both *in vivo* and vitro.

Hematopoietic Cells

Poster Board Number: T-2088

ERYTHROPOIETIC POTENTIAL IN HUMAN CORD BLOOD AND ADULT PERIPHERAL BLOOD FOR TRANSFUSION

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Blood transfusion is the process of receiving blood products into one's circulation intravenously. There are many patients around the world whose survival depends on safe blood transfusion. Nevertheless blood transfusion saves lives, but the transfusion of donated unsafe blood puts lives at risk because of HIV, hepatitis or other infections. As a countermeasure, hematopoietic stem cells from several human organs can be used for certain therapeutic uses as alternative safe blood source. There are many productive manuals to obtain mature RBCs from CD34+ hematopoietic stem cells via *in- vitro* culture. Among them human cord blood (CB) and adult peripheral blood (PB) are common adult stem cells for allogeneic transplantation. Owing of their largest, easily availability, addition of which contains large amounts of stem cells can be used to induce RBC maturation and transplantation. Our present study focused on comparison of CB and PB derived stem cell characterization and function test on *in-vitro* culture system. For experiment

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we obtained five cases of each CB and PB from normal human with informed consent. Selected CD34+ cells were cultured and characterized by common Giemsa staining, flow cytometery assay, quantitative PCR and hemoglobin electrophoresis; we also confirmed their functions using Hemox Analyzer. Our results from the study clearly reports that, CB and PB derived CD34+ hematopoietic stem cells have similar character in same culture conditions, however they show definitely differences in various gene expression level and final hemoglobin development. The results support the idea that CB and G-CSF mobilized PB possess different erythropoietic potential *in-vitro* culture system wiliest they express nearly same characters in some test events.

Poster Board Number: T-2089

CLINICAL OBSERVATION OF 24 CASES OF PATIENTS UNDERGOING AUTOLOGOUS PURIFIED BLOOD STEM CELL CD34+ FOR TREATMENT OF ANKYLOSING SPONDYLITIS

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Background: Ankylosing spondylitis (AS) is one kind of chronic and progressing disease which can relate to the central axis joints and cause arthropathy and mainly affect pelvic sacroiliac joint, vertebral column joint and latero-vertebral tissus and is very difficult to be restrained. Objectives: In order to find a new way to treat AS, we use purified autologous peripheral blood stem cell CD34+ as treating method and evaluate its effect. Methods: From November 2008 to October 2011, we collected 24 patients with AS, whose conditions were all consistent with the New York AS diagnostic criteria in 1984. We got the agreement with patients and his or her family. There were19 male and 5 female patients. The patients' ages were 20-40, median 31.5. The courses of disease had been 3 months to 25 years. In the 24 patients, 20 cases were HLA-B27 positive, 4 cases were HLA-B27 negative, and 15 cases had been treated with methotrexate, sulfasalazine, diclofebac sodium without good effect before our treatment. (1) The preparation of purified autologous peripheral blood stem cell CD34+: We added recombined human granulocyte stimulating factor(G-CSF, made in Northern China) 150μg-300μg/d for 3 days, hypodermic. After G-CSF mobilizing, using COBE machine, we collected peripheral blood 100ml, then from the blood purified CD34+ stem cells by CliniMACS machine, made the CD34+to (1.0-10.6) ×107, and the positive rate 85 per cent tested by flow cytometry.(2)Treatment method: Took the purified CD34+ stem cells about (1-3)×107 ,volume 40ml,infused separately into the patients' double sides sacroiliac joint cavities (each for 5ml) and along the patients' two sides of erector muscle of spine in punctiform injected about 30ml . After treatment we instructed them to enhance article functional training. Except 5 patients were still orally administrated small amount of diclofenac sodim, sulfasalazine,all the other patients did not use any medicine. Results: Following up to visit the patients for 3-26 months, we found the patients that mainly in the cervical part, the waist,the sacroiliac joints appeared easement of pain, in vertebral column the active limits were expanded and the consciousness turned much better than before. Before treatment, 24 cases of patients BASDAI:1.8-13.8, mean value 4.46±2.66, BASFI:8-78 points,mean value 28.67±18.25. After treatment , following up for 3 months, we found that the 24 cases of AS patients BASDAI:0.4-4.5, mean value 2.47±1.30; BASFI:2-66 points,mean value14.29±13.59. We applied EXCELT-TEST software procedure to do matched-pairs analysis, all the P values were less than 0.01, post-treatment was obviously better than prior-treatment.Imaging test of the vertebral column, sacroiliac joints did not show clearly change between post- treatment and prior-treatment. Following up for more than half a year, 14 cases of patients, we found the above index was still stable. Following up for more than a year, we found, 4 cases patients that the disease states were still in stable phase, 20 cases HLA-B27 positive without turning to negative. After treating all the 24 patients for 3 months, we tested their blood routine, liver function and kidney function all appearing normal and no other bad reaction. Conclusion: That using purified autologous peripheral blood stem cell CD34+ to treat the patients with AS could relive the patients' clinical symptoms, improve their life quality, and would be a safe and effective method for treating AS.

Poster Board Number: T-2090

DETERMINING THE EFFICACY OF HUMAN-TRIM5-CYP IN INHIBITING A BROAD SPECTRUM OF HIV VIRUSES WHEN STABLY EXPRESSED IN CD34-DERIVED MACROPHAGES

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Hematopoietic stem cells genetically modified to express anti-HIV genes can repopulate the immune system, replacing HIV target cells such as CD4+ T lymphocytes and macrophages. If such cells express therapeutic genes that halt early steps in the viral life cycle, HIV replication can be suppressed prior to reverse-transcription and integration. The HIV host restriction factor, huTrimCyp, was recently developed and does not occur naturally in humans [1]. It is believed that huTrimCyp inhibits HIV shortly after viral entry by binding to capsid proteins and sequestering them for degradation [2]. My preliminary data indicates that T-cell lines transduced with a lentivirus vector expressing huTrimCyp showed no short term or long term cytotoxicity, as well as exhibited a stable gene expression for a period greater than six weeks in vitro. Furthermore transduced T-cell lines showed an inhibition of R5- tropic HIV strain greater than 5 fold when compared to cells expressing ZsGreen alone as determined by flow analysis of HIV-reporter infected MOLT4 T-cell line. To that end, I have initiated experiments in CD34+ hematopoietic stem cells in order to determine the feasibility of pursuing huTrimCyp as an anti-HIV gene therapy candidate *in vivo*. I will also investigate the ability of vector transduced HIV target cells (e.g. CD4 T lymphocytes, macrophages) to resist infection and inhibit viral replication in vitro, using X4- and R5-tropic strains of HIV. 1.Neagu, M.R. et al. Potent inhibition of HIV-1 by TRIM5-cyclophilin fusion proteins engineered from human components. J. Clin. Invest. 119, 3035-3047 (2009). 2.Nakayama E. E. et al. Anti-retroviral activity of TRIM5. Rev. Med. Virol.; 20: 77-92 (2010)

Poster Board Number: T-2091

CHARACTERIZATION OF DENDRITIC CELLS INDUCED FROM HUMAN CORD BLOOD HEMATOPOIETIC STEM CELLS IN THE SERUM-FREE CONDITION

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Dendritic cells (DCs) are the most powerful antigen presenting cells (APCs) and play a pivotal role in initiating the immune response, which differentiated from CD133+ hematopoietic stem cells (HSCs). Hence, we used the *ex vivo* expanded of hematopoietic stem cells as a source of DCs, and developed the optimal DCs induction medium. In the previous study, we had developed a serum-free he-

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matopoietic stem cells expansion system (SF-HSC medium), HSCs could expand in SF-HSC medium reaching 30-fold within one week. According to the past researches, several cytokines, especially SCF, Flt-3 ligand, IL-1β, GM-CSF and TNF-α, have been identified as essential factors to induce and differentiate HSCs into DCs. Moreover, we tested the basal media (IMDM, RPMI-1640, M-199, α-MEM, H3000, DMEM/F12 and DMEM) and twenty-five kinds of serum substitutes combined with the various concentration of cytokines to finalize the optimal DC induction medium. Finally, we confirmed the function and maturation of DCs by the assays of the mixed lymphocyte reaction (MLR), the ability of endocytosis, specific cytokines of secretion and the stimulation by lipopolysaccharides. When DCs become mature (mDCs), the specific surface markers of mDCs would change (CD1a, CD11c, CD14, CD40, CD80, CD83, CD86, HLA-DR, CD45 and CD34), the ability of endocytosis would be more complete and the ability of stimulation would increase when co-cultured with CD3+ T cells. These results showed that DCs derived from the serum-free expanded CD133+ HSCs exhibited both characteristics and functions of DCs. Therefore, we believed that combination of HSCs serum-free expansion medium and DCs induction medium would generate large amounts of functional DCs and would be a promising cell source for the basic research and translation media in the near future.

Poster Board Number: T-2092

CELLULAR IMMUNE RESPONSE FOLLOWING HUMAN FETAL LIVER-DERIVED STEM CELLS TRANSPLANTATION IN CASE OF DECOMPENSATED LIVER CIRRHOSIS

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Liver transplantation is the only effective treatment for decompensated liver-cirrhosis. Several factors, such as non-availability of donors, operative-risks, complications associated with rejection, usage of immunosuppressive agents, and high cost of treatment, make this strategy available to only a few people. Hepatic progenitor stem cell transplantation (HSCT) using human fetal liver-derived stem cells have been shown to be a good alternative to manage end-stage liver diseases. In this retrospective study, we investigated safety and efficacy of HSCT by monitoring the T-cell, NK-cell and cytokines which play major role in cellular immune response and rejection of chronic decompensated liver cirrhosis patients. A total of 5 patients with decompensated liver cirrhosis were enrolled in the study.After giving human fetal liver-derivedEPCAM positive cell transplantation,T-cell (CD3, CD4 and CD8),NK-cells (CD16)by flowcytometry and cytokine-levels (IL2, TNFβ, IFNα, INFβ and IFNγ) by ELISA were monitored four times within a month. Present study demonstrated that after HSCT patient showed marked clinical recovery and decline in the MELD score and there was no significant variation found in cell mediated response and cytokine levels between pre and post transplantation.Hence this preliminary study demonstrated human fetal liver- derived EPCAM positive stem cell transplantation is safety for end stage liver cirrhosis.

Poster Board Number: T-2093

INTEGRATION-FREE HUMAN INDUCED PLURIPOTENT STEM CELLS FROM PERIPHERAL BLOOD MONONUCLEAR CELLS

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Human induced pluripotent stem cells (iPSCs) can be derived from somatic cells by ectopic expression of key factors and are highly similar to human embryonic stem cells (ESCs). This discovery led to the generation of patient-specific iPSCs which have unprecedented value for regenerative medicine, disease modeling and drug screening. However, iPSC derivation commonly utilizes skin fibroblasts, which involves donor skin biopsy and is time-consuming. In addition, human skin fibroblasts may have been exposed to UV irradiation and have increased risk of harboring genetic abnormalities. Sampling of blood is one of the least invasive but most routinely performed clinical procedures, making it an ideal cell source for reprogramming. Additionally, blood cells that are derived from hematopoietic stem cells (HSCs) in marrow before being released into circulation may be less exposed to environmental mutagens. Of the types of blood cells used for reprogramming, CD34+ blood cells come to mind first because they are enriched for HSCs and have high proliferation potential. However, CD34+ blood cells are very rare and the purification is cumbersome. Another concern is that they may not be feasible for certain diseases like aplastic anemia in which patients have a shortage of CD34+ blood cells. Thus the generation of iPSCs from a small amount of peripheral blood mononuclear cells (PB MNCs), with no need of fractionation, would be a big leap. iPSCs can be established from terminally differentiated circulating T cells expanded from one milliliter of whole blood. However, the pre-existing T cell receptor rearrangement may limit iPSC differentiation ability to the full spectrum of cell linages. Other researchers also found mice generated from T cell somatic nuclear transfer are prone to lymphomagenesis, which may complicate the usage of T cell as a reprogramming source. To find an alternative, we chose a condition to stimulate myeloid-erythroid growth from un-fractionated MNCs. After 8-9 days of culture, cells were under proliferative status, showed erythroblast antigen expression and were enriched for colony-forming erythroid progenitor cells (erythroblasts). To assess the reprogramming potential of expanded erythroblasts, we first compared them with cord blood (CB) CD34+ cells using either retroviruses or episomal vectors. Similar high efficiencies of iPSC derivation were achieved from either cell type. Hundreds of TRA-1-60+ colonies can be identified from one million starting cells in just two weeks by either type of vectors. To be more definitive, we reprogrammed CD34+ cells and expanded erythroblasts from the same adult donor by episomal vectors. The data showed reprogramming efficiencies were in similar levels although they are ~50x less than CB cell types. iPSCs derived from two starting blood cell sources are both indistinguishable from ESCs with respect to cell morphology, expression of surface markers, and the ability to differentiate in vitro and *in vivo*. Detailed analysis also indicated they are integration-free. Characterization of gene expression profile, epigenetic signature and re-differentiation capability to blood cells is currently underway and will be discussed during the meeting. Taken together, our data suggest that erythroblasts from a small volume of PB MNCs are suitable for reprogramming by integration-free episomal vectors, and may provide an advantage for generating GMP-compliant iPSC lines for clinical applications.

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Poster Board Number: T-2094

FEV CONTROLS HEMATOPOIETIC STEM CELL DEVELOPMENT VIA FOXC1-ERK SIGNALING CASCADE

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Production of transplantable haematopoietic stem cells (HSCs) in vitro either from embryonic or induced pluripotent stem cells has not yet been successful. Therefore, it is critical to fully understand molecular mechanisms of HSC development *in vivo*. Recent evidence suggests that ETS transcription factors play important roles in HSC development and functions. Fev (Fifth Ewing Variant) is a member of the ETS family and required for the differentiation and maintenance of serotonergic neurons in zebrafish and mammals. However, its role in HSC development remains to be explored. Here we show that Fev is an important regulator of HSC development in vertebrates. In fev-deficient zebrafish embryos, HSCs were compromised and fewer T cells were found in the thymus. Genetic analysis and chemical treatment experiments support a mechanism whereby Fev regulates HSC development through a novel Foxc1-ERK pathway. Moreover, experiments with human cord blood show that Fev is also required for the maintenance and expansion of primitive HSCs/progenitor cells in human, indicating its conserved role in higher vertebrates. Finally, we demonstrated that Fev function in HSCs is distinct from its role in serotonergic neurons in both zebrafish and mouse systems. Our data indicate that Fev-Foxc1-ERK signaling is essential for haemogenic endothelium-based HSC development.

Poster Board Number: T-2095

HUMAN BONE MARROW NON-MESENCHYMAL MONONUCLEAR CELLS INDUCE TYROSINE HYDROXYLASE EXPRESSION IN SH-SY5Y CELLS IN A TIME-DEPENDENT MANNER

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Background: Excluding mesenchymal stromal cells, the mononuclear cell fraction of the bone marrow composes more than 99% of total cellular components in the bone marrow. This fraction contains both hematopoietic and nonhematopoietic cells, which have been shown to secret trophic or growth factors necessary for neurogenesis. This study aimed to investigate the effect of the non-mesenchymal mononuclear cell fraction on the biochemical adaptation of human SH-SY5Y neuroblastoma cells. Methods: The mononuclear cells were isolated from the bone marrow of three human donors. After 72 hours of cell plating, non-adherent cells were collected and plated for another 24 hours. Thereafter, non-adherent bone marrow mononuclear cell-conditioned medium (BMMC-CM) was collected. SH-SY5Y cells were divided into three groups: cells cultured in the BMMC-CM, cells cultured in basal media with 10 μM retinoic acid (RA), and cells cultured in basal media (control). Cells were cultured for 4, 7, and 10 days, and media were changed every other day. Tyrosine hydroxylase (TH) expression in SH-SY5Y cells was measured using real-time PCR and western blotting. Results: TH mRNA expression was significantly increased at day 4 in cells cultured in BMMC-CM, compared to day 7 and 10 ($p<0.01$ and 0.001, respectively). In contrast, cells cultured in basal media with RA had a significant TH MRNA level at day 10, compared to day 4 and 7 ($p<0.001$ for both). At the protein level, TH was undetectable at day 10 in cells cultured in BMMC-CM; while in RA-treated cells, TH was significantly increased at day 7 and remained high at day 10. Conclusion: Non-mesenchymal mononuclear cells from the bone marrow have a potential in induction of functional differentiation in SH-SY5Y cells, but the effect is time-dependent.

Poster Board Number: T-2096

DEPLETION OF HUMAN HEMATOPOIETIC STEM CELLS WITH A HUMANIZED ANTI-HUMAN CD117 ANTIBODY: A NON-TOXIC CONDITIONING REGIMEN FOR ALLOTRANSPLANTATION

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Allogeneic hematopoietic cell transplantation (AHCT) is an important treatment modality for many malignant and non-malignant hematologic disorders. Most patients who undergo AHCT are prepared with cytotoxic chemotherapy and/or radiation to eliminate barriers to donor engraftment. For patients with malignancies, toxic conditioning agents also play a role in eradicating residual cancer cells. For patients with non-malignant disorders, conditioning regimens with no or little toxicity would be preferable. Our group previously demonstrated that an anti-CD117 (c-Kit) monoclonal antibody (mAb) eliminates endogenous hematopoietic stem cells (HSC) and facilitates engraftment of purified donor HSC in a mouse model of severe combined immunodeficiency (SCID) (Czechowicz et al., Science, 2007). Patients with SCID are highly susceptible to life-limiting infections, and while AHCT is the only effective therapy for most forms of SCID, treatment-related sequalae provide an urgent need to improve methods for achieving donor HSC engraftment. Based on our pre-clinical model, we believe anti-CD117 mAb therapy would be an ideal, minimally-toxic conditioning regimen for AHCT in SCID children. To that end, we identified a humanized anti-human CD117 mAb (anti-hCD117) as a potential candidate for clinical application in AHCT preparation. When human cord blood or bone marrow derived HSC (Lin-CD34+CD38-CD90+CD45RA-) were cultured in serum-free media with Flt3 ligand, stem cell factor, thrombopoietin, IL-3, and IL-6, profound inhibition of cell division was observed in the presence of 0.1 - 100 ug/mL anti-hCD117. Anti-hCD117 also inhibited HSC growth and differentiation in a methylcellulose colony assay. To assess *in vivo* activity of the anti-hCD117 mAb, we determined mAb pharmacokinetics in NOD/scid/IL2Rγ-/- (NSG) mice. We determined that doses between 0.5mg/kg and 3mg/kg provided appropriate maximal concentrations (5.8 - 38.3 ug/mL) and half-lives (10.4 - 12.4 days) for pre-clinical modeling. NSG mice were hematopoietically humanized on day 2 of life via facial vein injection of 2000-4000 human HSC following conditioning with 100cGy. After permitting hematopoietic stabililzation for 6 months, we evaluated xenochimerism in the peripheral blood (PB) and bone marrow (BM; via femoral aspirate in anesthetized mice). Overall human leukocyte chimerism in the BM of 32 mice was a median 4% (range 0.1 - 51%) with median differential composition: 13% (0-96%) B lymphocytes, 16.4% (0-95%) T lymphocytes, 0.02% (0-1%) NK cells, 2.5% (0-36%) monocytes, and 3.7% (0-49%) granulocytes, indicating engraftment of pluripotent HSC. After treatment with 3mg/kg or 0.5mg/kg anti-hCD117 IV, mice were depleted of total human chimerism a median 60% (35-100%) in the PB and 100% (84-100%) in the BM at 6 weeks after treatment, with >99% depletion of myeloid chimerism in both compartments. Human HSC and progenitor cells (Lin-CD34+CD117+) in the bone

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marrow comprised 0.4% (0-1.7%) of cells prior to anti-hCD117 treatment, and this decreased to 0% (0-0.1%) 6 weeks after treatment. Addition of alemtuzumab (anti-CD52) facilitated removal of human lymphoid cells not eradicated by anti-hCD117, but was not required for human HSC depletion. These studies will lead the way to minimally toxic AHCT for SCID children, and in a broader view, to the application of targeted biological therapies that deplete endogenous stem cells and facilitate their replacement with allogeneic or gene-corrected stem cells.

Poster Board Number: T-2097

CD34$^-$LIN$^-$ HAEMATOPOIETIC HUMAN ADULT STEM CELLS FROM BONE MARROW DONORS ARE POTENT SOURCES FOR THE GENERATION OF DENDRITIC CELLS

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Background & Objectives: Haematopoietic stem cells (HPSCs) transplantation is the treatment of choice for patients with refractory leukemia. Addition of adjuvant vaccines may lead to eradication of minimal residual leukemia and prevention of relapse. Most of the clinical and experimental protocols have used undifferentiated bone marrow or CD34+HPSC for the differentiation of dendritic cells (DCs) but no study conducted so far in human, investigated the CD34-HPSC as a source for the generation of DCs. Consequently, we hypothesized that CD34-Lin-HPSCs can be a valuable source for the generation of an effective vaccine when the patients encountered any relapses. Materials & Methods: CD34+ and CD34-Lin-HPSCs in bone marrow (BM) samples were sorted from 10 normal donors by flow cytometry. Aldehyde dehydrogenase (ALDH) activity was detected along with other 4 the expression of a series of haematopoietic antigens: CD34, CD38, CD45, CD105. Subsequently, a cocktail of different cytokines [GM-CSF, TNF-α and IL-4] was used to differentiate the DCs and a subpopulation of DC expressing CD14-CD1a+ was investigated. Moreover, their allogeneic stimulatory capacity was tested by a mixed lymphocytes reaction (MLR). Results: CD34-Lin-HPSCs were characterized and their haematopoietic capacity was measured by the differentiation of DC. DC expressing CD1a+CD14- found to co-express surface molecules such as CD86, CD80, CD83, CD40, HLA-DR. furthermore, they were found to stimulate allogeneic T cells when tested by MLR Conclusions: Our study demonstrated that CD34-Lin-HPSCs are efficient source for the generation of DCs expressing CD1a+CD14 and their antigen presenting ability were comparable to their counterparts from CD34+Lin-HPSCs and hold promises in immunotherapy modality.

Poster Board Number: T-2098

LONG-TERM COMPARATIVE STUDY ON THE ENGRAFTMENT OF HUMAN HEMATOPOIETIC STEM CELLS IN SHEEP

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Background: *In utero* transplantation (IUT) of hematopoietic stem cells (HSCs) has been pursued as a treatment for congenital hematologic and genetic disorders. Although the engraftment of HSCs following IUT has been achieved, the levels of donor-cell engraftment have been too low for the treatment of most target diseases. In order to achieve clinically relevant levels of HSC engraftment, we have examined two methods in the setting of sheep IUT. One is the transduction of HSCs with the HoxB4 gene before transplantation, ie, the expansion of HSCs. The other is the myeloablation of recipients with busulfan (BU), ie, the expansion of niche. Here, we report long-term follow-up data regarding the engraftment of human HSCs in the two (HoxB4 and BU) groups. Methods: We performed IUT of human cord blood (CB) CD34+ cells (0.32 to 2.4 million) into the liver of fetal sheep at 45-49 gestational days (full term, 147 days) at which the immune system is premature. In the HoxB4 group (n = 4), human CB CD34+ cells were transduced with HoxB4 by the P gene-defective Sendai virus vector for 4 days, and the cells were transplanted into the fetuses. In the BU group (n = 4), BU at 3 mg/kg (calculated by maternal body weight) had been intravenously administered to pregnant ewes at 6 days before performing IUT of intact (neither transduced or cultured ex vivo) human CB CD34+ cells. The BU dose and the timing of administration with BU were selected in order to result in the desired outcome. As a control group (n = 6), we transplanted intact human CB CD34+ cells without BU administration. The engraftment of human hematopoietic cells in the lambs post-IUT was quantitatively evaluated by PCR of colony-forming units (CFUs) in the bone marrow. Results: The two methods (the HoxB4-transduction and BU-conditioning) similarly enhanced short-term engraftments of human HSCs in sheep (up to 3% in terms of the bone marrow CFUs for 5 months post-IUT). At further time points, human CFUs were not detectable either in the BU or control group. However, in the HoxB4 group, they were still detected in 2 out of the 4 lambs at 15 months post-IUT (1-2%), and in 1 out of the 4 lambs at 40 months post-IUT (2%). No leukemia developed in any of the sheep in the HoxB4 group at present (at 40 months post-IUT) in our study, unlike high incidence of leukemia in other large animal studies using a HoxB4-expressing retroviral vector (Zang et al, JCI 2008;118:1502-1510). Conclusion: In the context of IUT, although the two methods enhance the short-term engraftment of human HSCs in sheep (for 5 months), the transduction of HoxB4 results in longer-term engraftment of those (up to 40 months) compared to the conditioning with BU. These results imply that a cell-autonomous action would be more effective than a non–cell-autonomous action for long-term engraftment of HSCs at least in sheep.

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Poster Board Number: T-2099

HUMAN LEUKEMIC STEM CELLS ESCAPE CHEMOTHERAPY BY FORMING SUBCLONES IN DISTICNT NICHES

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Failure to eliminate leukemic stem cell (LSC) is presumed to be the reason why leukemia relapses after chemotherapy. The mechanism in which LSC escapes chemotherapy remains much unknown. It has been demonstrated in the previous studies that LSC develops from pre-leukemic stem cell (pre-LSC) and continually evolve into multiple sub-clones in concert with disease progress (Hong D, et al. Science 2008; Ford A, et al. JCI 2009; Anderson K, et al. Nature 2011). To reveal in the current study how does LSC form sub-clone in the bone marrow niches and escape chemotherapy, the xenograft leukemic model was established by transplanting acute lymphoblastic leukemic (ALL) cells into sub-lethally irradiated NOD-SCID IL2Rγ^{null} mice or anti-CD122 antibody-treated NOD-SCID mice. By applying dynamic technologies of ex-vivo imaging and immunoassaying, GFP-labeled leukemic cells were observed to home into the endosteal niche in the recipient bone marrow in 24h after intravenous injection and clonally grow. When the recipients presented clinically manifest disease, these mice were treated with chemotherapy medicines, Ara-C or/and Daunorubicin, for two to seven days. Majority of leukemic cells in the bone marrow were killed and consistently minimal cells resided in the BM niches. Interestingly, we observed that LSC exploited or created distinct niches for refuge in response to chemotherapy. Leukemic cells preferentially usurped the normal HSC niche given it was still intact, whereas leukemic cells created an abnormal stromal niche when the normal HSC niche was destroyed by leukemic cell infiltration. And the process was associated with the interaction between leukemic cells and chemotherapeutic drugs. The residual leukemic cells in the stromal niches isolated from the recipient BM were capable to reconstitute leukemia in the recipients of serial transplantation, thus were proved to be LSCs. By analyzing the biopsy samples of ALL patients who only achieved partial remission after induction chemotherapy, the abnormal stromal niche was observed surrounding leukemic cells and associated with poor prognosis. Therefore these studies have implication for therapeutics of leukemia depending on LSC eradication.

Poster Board Number: T-2100

STIMULATION OF POOLS OF MURINE HEMATOPOIETIC AND INTESTINAL STEM CELLS BY PERORAL INTAKE OF NANJDIAMONDS COMPLEXES WITH THE CONDITIONED MEDIUM FROM ALLOGENIC AND XENOGENIC MESENCHYMAL STEM CELLS CULTURE

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Nanodiamonds with the sizes of 5-10 nm have been intensively studied lately as prospective agents for the anticancer drugs delivery into tumors. A possibility of nanodiamonds application for the delivery of other biologically active substances to normal tissues is not excluded, either. We have found that peroral intake of a suspension containing complexes of nanodiamonds with the conditioned medium (CM) from the cultures of mesenchymal stem cells (MSC) by F1(CBAxC57Bl/6) mice activates the pool of hematopoietic stem cells (HSC). This can be observed by the method of spleen endocolonies after the total irradiation (γ-rays of Co^{60}) of animals in sublethal doses. We assume that the complexes of nanodiamonds with CM taken by mice may arrive into the HSC "niches" in the hematopoietic tissues and activate (by yet unknown way) HSC being mainly in the state of proliferative rest. This may result in the temporary increase of the HSC pool, as it is observed for a large group of radioprotective preparations with the "biological mechanism of action". We also studied the radioprotective effect of the complexes of nanodiamonds with CM from allogenic and xenogenic MSC cultures by the 30 days post-irradiation survival test of mice undergone the lethal dose of γ-irradiation. The same stimulation effect of the perorally-taken complexes of nanodiamonds with CM was also revealed by the test of "microcolonies" production by the intestinal stem cells of lethally irradiated mice and by the survival test of animals during development of the "intestinal form" of radiation death. Since the experimentally observed radioprotection effects are undoubtedly accounted for by the stimulation of regenerative activity of adult stem cells, then the task of primary importance is to study possible effects of such complexes using other nonradiation tests, in particular, using their influence on the stem cell-dependent efficiency of reparative processes accompanying various types of damages of different organs and tissues.

Poster Board Number: T-2101

CLONAL EXPANSION AND LINEAGE BIAS OF MOUSE HEMATOPOIETIC STEM CELLS ARE INDUCED BY PRE-TRANSPLANTATION CONDITIONING

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Hematopoietic stem cells (HSCs) are identified as cells capable of reconstituting the entire hematopoietic system upon transplantation into lethally irradiated recipients. Irradiation conditioning is used in the vast majority of HSC studies to facilitate HSC engraftment by depleting the recipient's hematopoietic system. While the massive loss of hematopoietic cells can be reconstituted by donor HSCs, it is unclear whether HSC regulation after this severe damage still resembles that under steady state conditions. Here, we compare HSC lineage commitment at the clonal level after lethal irradiation mediated transplantation, after unconditioned transplantation, and after c-kit antibody clearance based transplantation, which eradicates hematopoietic progenitor cells while leaving other hematopoietic cells intact. Clonal tracking of HSCs was performed using our recently developed single cell tracking system based on genetic barcoding and high-throughput sequencing. This experimental system offers high throughput and high sensitivity that allows for the direct examination of HSC clonality and for tracking the low numbers of HSCs that engraft after unconditioned transplantation. Our data suggests that HSCs are regulated differently after conditioned transplantation and after unconditioned transplantation. After unconditioned transplantation, all donor-derived HSC clones equally supply the peripheral blood with granulocytes and B cells, while only a small subset of HSCs supplies T cells. In contrast, after irradiation mediated transplantation and after ckit antibody clearance based transplantation, a small subset of engrafted HSC clones dramatically expands to supply the majority of granulocytes,

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B cells and T cells. This clonal expansion occurs at every step of the HSC lineage commitment process at week 22 post transplantation. Moreover, this expansion is unbalanced between myeloid and lymphoid lineages. In irradiated mice and ckit antibody treated mice, HSC clones exhibit differential contributions to granulocytes and B cells, a phenomenon previously identified as "lineage bias". However, this myeloid versus lymphoid lineage bias is not present after unconditioned transplantation. In addition, it is not exhibited at the progenitor level in irradiated mice. In these mice, HSC clones that are balanced at the progenitor level exhibit lineage bias at the mature blood cell stage. This indicates that lineage bias is induced by clonal expansion downstream of the oligopotent progenitors. In order to determine whether clonal expansion and lineage bias involve HSC clones that expand immediately after transplantation, we examined granulocytes in the peripheral blood at week 4 and week 8 after irradiation mediated transplantation. These early-expanded HSC clones are under represented by HSC clones that undergo expansion at week 22 and are over represented by HSC clones with lymphoid bias. This suggests that lineage bias induced by pre-transplantation conditioning may be related to time dependent clonal expansion post transplantation. In summary, HSCs exhibit clonal expansion and lineage bias after conditioned transplantation but not after unconditioned transplantation. These two induced features are associated with each other throughout the HSC lineage commitment process and are intertwined over time following conditioned transplantation.

Poster Board Number: T-2102

IGF2 ACCELERATE ERYTHROID DIFFERENTIATION IN THE MOUSE YOLK SAC

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In vertebrates, both primitive and definitive hematopoiesis first occurs in yolk sac (YS), which consists of hematopoietic cells, capillary blood vessels, and endodermal cells. Although hematopoietic cell fate is extrinsically determined by niche cells, little is known about its regulation at YS. There was no specific marker to recognize YS capillary blood vessel. We successfully found that lymphatic endothelium hyaluronan receptor (Lyve-1) is expressed on YS capillary blood vessels (micro-vessels), but not on arteries and veins (non-micro-vessels). Single YS cells at E10.5 were prepared by collagenase treatment and analyzed by flow cytometry. Ter119+ and/or CD45+ cells as "hematopoietic cells", Ter119-/CD45-/CD31+/Lyve-1+ cells as "micro-vessels", Ter119-/CD45-/CD31+/Lyve-1+ cells as "non-micro-vessels" and Ter119-/CD45-/CD31-/Lyve-1- cells as "unclassified cells", which are likely endodermal cells we re sorted out, following gene expression of hematopoietic cytokines was examined by real-time PCR. Among several cytokine genes, Insulin-like growth factor 2 (Igf2) was predominantly expressed at E10.5 YS. In addition, expression level of Igf2 in unclassified cells was 7.0, 9.6 and 15.9 times higher than micro-vessels, non-micro-vessels and hematopoietic cells, respectively. To further investigate the function of Igf2 in YS hematopoiesis, neutralizing antibody against Igf2 was added *in vitro* culture of YS cells for 6 days. The production of erythroid progenitor cells (c-Kit+/CD71+), erythroblasts (CD71+/Ter119+) and mature erythrocytes (CD71-/Ter119+) were decreased after inhibition of Igf2. Taken together, endodermal cells likely secrete Igf2, which functions in erythroid differentiation in YS.

Poster Board Number: T-2104

ALCAM MEDIATED CELLULAR INTERACTION REGULATES MOUSE HEMATOPOIETIC STEM CELL REPOPULATION AND SELF-RENEWAL

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Alcam, which encodes the activated leukocyte cell adhesion molecule (CD166), is a cell surface immunoglobulin superfamily member mediating homophilic adhesion as well as heterotypic interactions with CD6. It has recently been shown that Alcam+ endosteal subset in the bone marrow contain hematopoietic niche cells able to support hematopoietic stem cell (HSC) activity. We use an Alcam null mouse model to assess the function of Alcam in HSC differentiation and self-renewal. We found that Alcam is highly expressed on the cell surface of long-term repopulating HSC (LT-HSC), multipotent progenitors (MPP), and is expressed on a subset of granulocyte/macrophage progenitors (GMP). The frequency and cellularity of bone marrow LT-HSCs are not altered in Alcam-deficient mice. We show that the serial-replating potential of Alcam-deficient LT-HSCs is reduced using a clonogenic colony-forming progenitor (CFC) assay. We examined the differentiation potential of individual phenotypic LT-HSC using an in vitro single-cell differentiation assay. Our results show that Alcam-deficiency leads to an enhanced granulocytic differentiation. We performed competitive repopulation assays *in vivo* followed by secondary transplantation. We show that Alcam-deficient cells show a transient engraftment enhancement, however, the engraftment is significantly lower upon secondary transplantation. These results suggest that the self-renewal capacity of Alcam-deficient HSC is compromised. We performed a limiting-dilution transplantation assay and determined that the frequency of long-term repopulating cells in Alcam-deficient bone marrow is significantly reduced. We further assessed the engraftment efficiency of limiting numbers of phenotypically purified LT-HSCs and show that the engraftment efficiency of Alcam-deleted LT-HSCs is significantly reduced compared to wild type LT-HSCs. We observed that Alcam-deficient progenitors are significantly more responsive to SDF-1 chemo-attractant. Collectively, these studies implicate Alcam mediated cell-cell interaction in the regulation of HSC self-renewal and migration.

Poster Board Number: T-2105

ANALYSIS OF HEMATOPOIETIC AND MESENCHYMAL STEM CELL MARKERS FROM MOUSE YOLK SAC IN DIFFERENT GESTATIONAL STAGES

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Yolk Sac (YS) is an embryonic attachment present during vertebrate development, responsible for embryonic feed during development, protein synthesis, phagocytic activity, material transfer and hematopoiesis. YS derived cells from dogs fetuses were cultured for a long term without plasticity lost in our lab. SV cells retain the ability to rapid proliferate and can be differentiated in blood cells, including lymphocytes, granulocytes, monocytes, erythrocytes and

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megakaryocytes. Thus, they can have a wide spectrum of applications, including rescuing or recovering of hematopoietic system, transgenic animal for blood and antibody production, vaccine and immunotherapy production and other applications. Therefore, in this project we aim to establish and characterize YS stem cells from mice in different gestational stages in order to analyze comparatively the pluripotency, mesenchymal and hematopoietic stem cell markers in these cells, morphology and behavior in cell culture. Swiss mice with 10, 15 and 19 days of gestation were euthanized and SV were collected in a tube containing PBS plus 5% of antibiotics. After washing, tissues were minced into very small pieces and submitted to four different culture conditions: 1) DMEM supplemented with 20% of FBS, 200 U/ml penicillin, 200 µg/ml streptomycin, 2) DMEM/F12 supplemented with 15% of FBS, 200 U/ml penicillin, 200 µg/ml streptomycin, 1% L-glutamine, 1% MEM NEAA, , 1% L-glutamine, 1% NEAA 10^{-4}M of β-mercaptoethanol; 3) DMEM high glucose supplemented with 7,5% FCS, 200 U/ml penicillin, 200 µg/ml streptomycin, 1% L-glutamine, 1% MEM NEAA, 10^{-4}M of β-mercaptoethanol; and 4) α-MEM supplemented with 15% of FBS 200 U/ml penicillin, 200 µg/ml streptomycin, 1% L-glutamine, 1% MEM NEAA, 10^{-4}M of β-mercaptoethanol. The cells were maintained in a humidified atmosphere of 5% of CO^2 at 37°C. Fragmented tissues attached in a cell culture dish and start detaching cells usually after 24 hours, differing just depending on the medium used. Also, the detached cells kept around tissues or migrate to a point farthest from the origin to form small colonies which begin to expand. YS cells were positive for CD45 and CD90 using flow cytometry assay. The YS cell culture using explants method was achieved satisfactorily by our group. YS cells express cellular markers for mesenchymal and endothelial progenitor cells, suggesting that this cell population could be used to stimuli angiogenesis protocols. Experiments with these cells are being conducted by our group, also using other animal species.

Poster Board Number: T-2106

EPIGENETIC REGULATION OF MOUSE HEMATOPOIETIC STEM CELL AGING

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Hematopoietic stem cell (HSC) Aging is a complex process linked to number of changes in gene expression and functional decline of self-renewal and differentiation potential. While epigenetic changes have been implicated in HSC aging, little direct evidence has been generated. DNA methylation is one of the major underlying mechanisms associated with the regulation of gene expression, but changes in DNA methylation patterns with HSC aging have not been characterized. We hypothesize that revealing the genome-wide DNA methylation and transcriptome signatures will lead to a greater understanding of HSC aging. Here, we report the first genome-scale study of epigenomic dynamics during normal mouse HSC aging. We isolated SP-KSL-CD150+ HSC populations from 4, 12, 24 month-old mouse bone marrow and carried out genome-wide reduced representative bisulfite sequencing (RRBS) and identified aging-associated differentially methylated CpGs. Three biological samples were sequenced from each aging group and we obtained 30-40 million high-quality reads with over 30X total coverage on ~1.1M CpG sites which gives us adequate statistical power to infer methylation ratios. Bisulfite conversion rate of non-CpG cytosines was >99%. We analyzed a variety of genomic features to find that CpG island promoters, gene bodies, 5'UTRs, and 3'UTRs generally were associated with hypermethylation in aging HSCs. Overall, out of 1,777 differentially methylated CpGs, 92.8% showed age-related hypermethylation and 7.2% showed age-related hypomethylation. Gene ontology analyses have revealed that differentially methylated CpGs were significantly enriched near genes associated with alternative splicing, DNA binding, RNA-binding, transcription regulation, Wnt signaling and pathways in cancer. Most interestingly, over 579 splice variants were detected as candidates for age-related hypermethylation (86%) and hypomethylation (14%) including Dnmt3a, Runx1, Pbx1 and Cdkn2a. To quantify differentially expressed RNA-transcripts across the entire transcriptome, we performed RNA-seq and analyzed exon arrays. The Spearman's correlation between two different methods was good (r=0.80). From exon arrays, we identified 586 genes that were down regulated and 363 gene were up regulated with aging ($p<0.001$). For the RNA-seq analysis, we focused first on annotated transcripts derived from cloned mRNAs and we found 307 genes were down regulated and 1015 gene were up regulated with aging ($p<0.05$). Secondly, we sought to identify differentially expressed isoforms and also novel transcribed regions (antisense and novel genes). To characterize the genes showing differential regulation, we analyzed their functional associations and observed that the highest scoring annotation cluster was enriched in genes associated with translation, the immune network and hematopoietic cell lineage. We expect that the results of these experiments will reveal the global effect of DNA methylation on transcript stability and the translational state of target genes. Our findings will lend insight into the molecular mechanisms responsible for the pathologic changes associated with aging in HSCs.

Poster Board Number: T-2107

PRE HSCS ARE LOCATED IN INTRA-AORTIC HEMATOPOIETIC CLUSTERS BEFORE HSC PRODUCTION IN THE MOUSE EMBRYO

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Hematopoietic Stem Cells (HSCs) are at the foundation of all blood cells. They are first detected in the dorsal aorta at day (E)10.5 of the mouse embryonic development. HSCs derive from specialized endothelial cells capable of hematopoietic cell production and therefore named hemogenic. HSCs most likely reside in Intra-Aortic Hematopoietic Clusters (IAHCs) that are attached to the endothelial layer of the aorta. The very low number of HSCs compared to the very high number of IAHCs, and the earlier appearance of the IAHCs during development raises several questions. Here, we performed an in-depth analysis of the IAHCs to determine their exact cell composition and function. We tested the hematopoietic potential of the IAHC cells isolated from the aorta of E10.0 embryos *in vitro*. We specifically isolated the IAHC cells after intra-aortic injection of antibodies against c-kit and flow cytometry sorting. c-kit^{high} IAHC cells were further sub-fractionated based on the differential expression of CD45 and Ly6A(Sca1)-GFP. The four populations (c-kit^{high} $CD45^{+\text{ or }-}$ $Ly6A^{+\text{ or }-}$) were tested for B lymphoid, erythroid and myeloid potentials. We found that IAHCs contain very few erythro-myeloid progenitors. Most progenitors are in the circulating blood and in the c-kit^{high} $CD45^{+}$ $Ly6A^{-}$ population (similarly to the progenitors found in the yolk sac, fetal liver and placenta). Moreover, no B progenitors were present in the IAHCs. We then tested *in vivo* the pre-HSC potential of the IAHC cells. Pre-HSCs, capable of long-term and multilineage reconstitution after injection into the liver of newborn recipients, are present in E9 embryos. Using this assay, we found that IAHC cells isolated from E10.0 embryos contain

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pre-HSCs at a time when no HSCs can be detected yet (as classically defined by their ability to reconstitute irradiated wild type adult recipient). Moreover, successful secondary transplantations into wild type adult recipients demonstrate the maturation of the pre-HSCs (IAHCs) into fully potent HSCs. In addition, pre-HSCs were c-kit^{high} CD45^{-} Ly6A$^{+ \text{ or } -}$ or c-kit^{high} CD45^{+} Ly6A^{+}. In conclusion, we demonstrate that IAHCs contain pre-HSCs capable of producing HSCs *in vivo*. Such pre-HSC maturation into HSCs might occur *in vivo* during the course of embryonic development and be part of the massive HSC increase observed at mid-gestation in the fetal liver. Our data highlight the different steps leading to the formation of engraftable, and thus therapeutically amenable, HSCs.

Poster Board Number: T-2108

JAK2 V617F SUBVERTS MOUSE HEMATOPOIETIC STEM CELLS BY INCREASING PROLIFERATION AND DIFFERENTIATION WHILE REDUCING LONG-TERM SELF RENEWAL CAPACITY

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Hematopoietic stem cells (HSCs) are a tightly regulated cell population ultimately responsible for providing the correct numbers and types of blood cells throughout life. Imbalances in this regulation can perturb normal blood cell production and result in malignancy. Clonal myeloproliferative neoplasms represent an early stage of such imbalances and identification of the highly prevalent JAK2 V617F mutation provides a powerful tool for observing the process of stem cell subversion. Recently, our group has generated a conditional knock-in mouse in which a single copy of the human JAK2 V617F is expressed under the control of the mouse Jak2 locus. These mice show modest increases in hemoglobin and platelet levels, reminiscent of a mild human JAK2 V617F-positive essential thrombocythemia, together with a 10% transformation rate to more severe disease (polycthemia vera or myelofibrosis). Surprisingly, both competitive and non-competitive whole bone marrow transplantation experiments revealed a significant decrease in JAK2 mutant stem cell activity, evidenced most strikingly in secondary transplantation studies. Here, we undertook experiments to characterize the HSC compartment and to understand the stem cell defect evidenced by whole bone marrow transplantations. The frequency of HSCs (EPCR+/CD150+/CD48-/CD45+) in JAK2 V617F mutant animals is reduced and individual HSCs have qualitative defects when studied in vitro and in transplantation experiments. Specifically, JAK2 V617F alters the balance of HSC fate choices in vitro and *in vivo*, skewing progeny toward differentiation and proliferation resulting in a loss of HSCs. In aged cohorts, HSCs from JAK2 V617F mice are not expanded and the relative proliferative advantage of their progeny is restrained, however the tendency to differentiate is maintained. Approximately 10% of mice progress to more severe disease, and together these data suggest that the JAK2 V617F mutation leads to HSC exhaustion, but exposes cells to the accrual of additional genetic lesions and eventual transformation.

Poster Board Number: T-2109

ROLE OF THE CLASP2 MICROTUBULE PLUS-END TRACKING PROTEIN DURING MOUSE ADULT HEMATOPOIESIS

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Hematopoietic Stem Cells (HSCs) are at the foundation of the hematopoietic system in vertebrates. They are multipotent and capable of self-renewal, which allow the constant production of all blood cell types throughout life without exhaustion of the HSC pool. HSCs reside in the bone marrow in specific areas or niches. HSC fate is controlled by the surrounding microenvironment via cell-cell or cell-extracellular matrix (ECM) contacts and by the production of growth factors. Therefore, attachment, migration and (a)symmetric division are very important for a proper HSC homeostasis. It is known that the microtubule (MT) cytoskeleton plays an important role in these processes. However, little is known about the organization and function of the MT network in HSCs. Mammalian CLASPs are MT plus-end tracking proteins that play essential roles in the local regulation of MT dynamics. To determine the function of CLASP2 in hematopoiesis, we have generated *Clasp2* knockout mice. Adult knockout mice are viable but develop a severe pancytopenia. We observed, after performing long-term transplantations, a strong HSC defect in the bone marrow of the knockout mice, which is partially due to defective homing of hematopoietic cells to the bone marrow. We also found that the MT network is impaired in the *Clasp2* knockout hematopoietic stem and progenitor (HSPCs), which results in a cell attachment defect *in vitro*, and possibly also *in vivo* in the bone marrow niche. Finally, we found that HSC-enriched populations sorted from the bone marrow of *Clasp2* knockout mice (Lin^{-}Sca-1^{+}c-kit^{+}) contain reduced mRNA levels of *c-Mpl* (which encodes the thrombopoietin receptor) and *Meis1* (transcription factor regulated by c-Mpl). Both factors are known to be essential for HSC maintenance. Altogether, we show that CLASP2-mediated stabilization of MTs is required at multiple levels for HSC activity, including homing, attachment, and signaling (via the transcription of essential HSC genes).

Poster Board Number: T-2110

POLYCOMB REPRESSIVE COMPLEX-1 COMPOSITION DETERMINES MOUSE HEMATOPOIETIC STEM CELL FATE

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Adult stem cells have the unique capacity to undergo both symmetric and asymmetric divisions, leading to their self-renewal or differentiation. A precise coordination of these cell fate decisions is essential to sustain tissue homeostasis. Here we show that different CBX-associated Polycomb Repressive Complex-1 (PRC1) complexes regulate the switch between self-renewal and differentiation divi-

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sions in hematopoietic stem cells (HSCs). Among all Cbx proteins, Cbx7 showed to be highest expressed in long-term HSCs, and its expression decreased upon differentiation. Overexpression of Cbx7 specifically induced self-renewal divisions of HSCs, and resulted in development of multiple leukemia-subtypes upon transplantation in mice. Abrogating its binding to methylated histon 3 or Ring1b, completely impaired the effect of Cbx7 on HSC self-renewal. Modulation of the PRC1 composition in HSCs via overexpression of Cbx4 or Cbx8, resulted in enhanced differentiation and subsequent HSC exhaustion.
Using global chromatin occupancy analyses (ChIP-seq) we found that two alternatives might account for the functional differences between Cbx7 and Cbx8. First, Cbx8 unique targets showed to be significantly enriched in genes involved in transcription, signal transduction, and apoptosis. Second, we did not find unique Cbx7 targets and Cbx7 showed to have lower chromatin binding affinity than Cbx8 in general. The binding of Cbx7 showed to be significantly higher than Cbx8 at only a few genomic positions. These correspond to transcription start sites of genes involved in transcription, cell cycle, and development. Together, our results show that the Cbx-subunit determines PRC1 target selectivity which provides a key molecular switch between HSC self-renewal and differentiation.

Poster Board Number: T-2111

HOMING OF BONE MARROW AND PURIFIED TRANSPLANTED STEM CELLS IN MARROW ABLATED MICE.

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Stem cell-based therapy has been one of the best documented approaches in regenerative medicine, promising cures for treatment of a multitude of diseases and disorders. The whole body irradiation of mice by lethal or sublethal doses represents a model for elimination of endogenous hematopoiesis and demonstration of eficcacy of transplantation with hematopoietic cells. Homing is a rapid process and it is the first and essential step in clinical cell transplantation. A special feature of the intravenously transplanted HSCs and HPCs is their migration from peripheral blood (PB) to bone marrow (BM), a process referred to as homing. In our experiments we have used positively selected bone marrow cells lin- /CD117+ cells that are presented less heterogenous than the whole bone marrow. We have performed a histochemical analysis of GFP+ cell engraftment in the recipient's tissues. Tissue analyses were performed on day 30 and day 70 after exposure to 9 Gy irradiation and transplantation of bone marrow cells or lin-/CD117+ (GFP+) cells. After 30 days in the bone marrow, the transplanted cells were frequently observed in the vicinity to the endosteum of bone trabeculi Large numbers of grafted cells entered the thymic parenchyma. Lot of GFP+ cells engrafted the lamina propria of small intestines and splenic parenchyma. On the other hand, GFP+ hematopoietic cells were rarely observed in the liver. After 70 days, numerous transplanted cells occupied the core of intestinal villi and splenic nodules in the splenic white pulp. In the liver, most GFP+ cells differentiated into hepatic Kuppfer cells. Transplanted cells differentiated into hematopoietic cells, lymphocytes and Kuppfer cells. We detected GFP+ gene expression in the recipient's tissues using qRT-PCR. Data confirm colonisation of recipient tissues with transplanted cells on day 30 and day 70 post-transplantation. After bone marrow transplantation, we detected increased levels of GFP+ in the spleen on day 30, and also in the thymus. On the other hand, transplantation of lin-/CD117+ cells resulted in the largest accumulation of engrafted cells in the spleen on day 30 followed by a transient decrease; next peaks were detectable on day 70 in the spleen and thymus. The bone marrow, small intestine and liver showed engraftment with GFP+ cells although their levels did not reach those observed in the spleen and thymus. Transplantation of lethally irradiated mice with bone marrow-derived cells supports hematopoiesis and leads to colonization of diverse organs with grafted cells. Engraftment efficiency depends on variety factors including number and type of transplanted cells.

Poster Board Number: T-2112

ROLE OF BMI-1 IN NORMAL MOUSE HEMATOPOIETIC STEM CELLS AND LEUKEMIC CELLS

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Hematopoietic stem cells (HSCs) are well-characterized stem cells, which are able to replenish cells of all lineages in the hematopoietic system. It is known that Bmi-1, as a core component in polycomb repressor complex 1, helps to sustain self-renewal and proliferation of HSCs. Bmi-1 was previously reported as negative regulator of *Ink4a-Arf* tumor suppressor gene locus, which is involved in the regulation of cell cycle in hematopoietic cells. Apart from the *Ink4a-Arf* locus, little is known about the cellular targets of Bmi-1, which presumably play crucial role in self-renewal and differentiation of HSCs. Moreover, aberrant expression of BMI-1 was observed in human leukemias, suggesting the deregulation of BMI-1 is highly associated with leukemogenesis, yet its function remains unclear. In this study, hematopoietic stem cells with $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ (LSK) phenotype were isolated from mouse bone marrow and were subjected to *Bmi-1* gene knockdown. Q-RT-PCR analysis showed that knockdown of *Bmi-1* gene leads to de-repression of $p16^{Ink4a}$ and $p19^{Arf}$ genes, without alteration of other cell cycle regulators and hematopoietic lineage genes, in LSK cells, suggesting that Bmi-1 has limited function on stem cell differentiation. Interestingly, cell cycle analysis showed that depletion of *Bmi-1* drives cells from G0/G1 phase to S phase, which overturns the effect of elevated $p16^{Ink4a}$ and $p19^{ARF}$ expression in blocking the entry to S phase, suggesting an alternative mechanism of cell cycle regulation is involved. Colony forming assay further demonstrated reduced colony forming ability of *Bmi-1* knockdown LSK cells, which indicates defects in their self-renewal ability. On the other hand, *Bmi-1* was over-expressed in HL-60 promyelocytic leukemia cells. Cell proliferation study demonstrated an increased in total cell number with a higher proportion of cells in S and G2/M phases. We also found that over-expression of *Bmi-1* increased $p14^{ARF}$ and $p19^{INK4A}$ gene expression, which explains the observed S/G2/M phase progression. Ongoing investigation is to find out the epigenetic regulation mediated by Bmi-1 on its target genes for the self-renewal pathway in both normal HSCs and leukemic cells. By revealing the functional role of BMI-1 in hematopoietic system, it provides insight into the development of novel therapeutic strategies which could potentially eradicate leukemic stem cells.

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Poster Board Number: T-2113

TRANSCRIPTOME ANALYSIS OF PRIMITIVE STREAK DURING HEMATOPOIETIC COMMITMENT

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The earliest adult mouse repopulating hematopoietic stem cells (HSCs) arise from the aorta-gonad-mesonephros (AGM) during E10.5 of gestation. To further elucidate the molecular mechanism of HSC development in the AGM, we studied the transcriptome of the primitive streak as it acquires hematopoietic potential. Primitive streak cells were isolated from E8.5 Brachyury (Bry)-GFP embryos via flow cytometry. These cells (Bry+Flk1-) are not yet committed towards the hematopoietic fate, as observed from the lack of hematopoietic cells when cultured on hematopoiesis-supporting OP9 stroma. However these Bry+Flk1- cells can be directed towards a hematopoietic (erythroid, myeloid and lymphoid) fate or a non-hematopoietic program by a reaggregation step in the presence of different culture conditions. By comparing the microarray-derived transcriptome profiles of these populations after reaggregation, we identified a group of genes that greatly increase in expression levels as the primitive streak acquires hematopoietic potential. Quantitative PCR analysis was used to narrow the list of candidate genes for further study. We are currently generating inducible knockdown and overexpression ES lines for one of the candidate genes, to identify its involvement and functional significance in hematopoietic development, which we hope to present in this poster.

Poster Board Number: T-2114

DOK2 REGULATES GLOBIN GENE EXPRESSION IN MOUSE EMBRYONIC ERYTHROPOIESIS

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Downstream of tyrosine kinase 2 (Dok-2) reportedly plays an important role in myeloid cell homeostasis particularly leukemia suppression and is also one of the novel signaling cascade proteins stimulated in the activated platelets. The Dok-2 gene has been obtained from our database in micro-array analysis of c-myb deficient embryos which impaired fetal liver erythropoiesis. As Hematopoietic Stem Cells (HSCs) differentiate into erythrocytes, Dok-2 gene expression was highest in the HSC (CD45+, Sca1+ and c-kit+) fraction, but declined in erythroid lineage, such as BFU-E, CFU-E, erythroblasts and mature erythrocytes, suggesting that Dok-2 gene is involved in erythroid differentiation. We also found that Dok-2 gene was down-regulated in both HSC and BFU-E fractions in heterozygous c-myb mutant mice as compare to wild type. To investigate the role of Dok-2 in erythroid differentiation, HSC and BFU-E fraction (CD45-, Sca1-, Ter119- and c-kit+) from fetal liver cells at 14.5 dpc were transfected with Dok-2 siRNA. After 24 hours of siRNA transfection, gene expressions of beta-major-, beta-minor- and epsilon-globins were up-regulated in BFU-E fraction, but slightly down-regulated in HSC fraction. Taken together, Dok-2 regulates globin gene expression during erythroid differentiation in the mouse fetal liver. Key words: Dok-2, Hematopoietic Stem Cells, Erythropoiesis

Poster Board Number: T-2115

SIRT1 IS DISPENSABLE FOR FUNCTION OF HEMATOPOIETIC STEM CELLS IN ADULT MICE

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SIRT1 is an NAD+-dependent histone deacetylase implicated in the establishment of the primitive hematopoietic system during mouse embryonic development. However, investigation of the role of SIRT1 in adult hematopoiesis has been complicated by high perinatal mortality of SIRT1-deficient mice (SIRT1-/-). Herein we perform a comprehensive *in vivo* study of the hematopoietic stem cell (HSC) compartment in adult SIRT1-/- animals and show that, apart from anemia and leukocytosis in older mice, production of mature blood cells, lineage distribution within hematopoietic organs and frequencies of the most primitive HSC populations are comparable to those of wild type littermate controls. Furthermore, we show that SIRT1-deficient bone marrow cells confer stable long-term reconstitution in competitive repopulation and serial transplantation experiments. Taken together, our results rule out an essential physiological role for cell-autonomous SIRT1 signaling in maintenance of adult HSC compartment in the mouse.

Poster Board Number: T-2117

ACTIVATED G_s SIGNALING IN IMMATURE OSTEOBLASTS ALTERS THE HEMATOPOIETIC STEM CELL NICHE IN MICE

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Adult hematopoiesis occurs primarily in the bone marrow space where blood cells closely interact with stromal niche cells, including osteoblastic lineage cells. Despite this close association, little is known about the specific roles of osteoblasts in supporting hematopoietic stem cell (HSC) function, and how conditions affecting bone formation influence hematopoiesis. Here, we use a constitutively-active engineered Gs-coupled G-protein coupled receptor (GPCR) to activate Gs-GPCR signaling in osteoblasts and assess how the concomitant increase in bone formation impacts HSC function and blood homeostasis. The Coll(2.3)+/Rs1+ transgenic mouse model of fibrous dysplasia of the bone showed a 5-15 fold increase in trabecular bone mass with near complete loss of the normal bone marrow cavity. The mice showed BM aplasia with progressive loss of up to 85% of HSC numbers and impaired megakaryocyte and erythrocyte development with defective recovery after myeloablation with 5FU. These blood phenotypes developed without compensatory extramedullary hematopoiesis. Surprisingly, the loss of HSCs occurs despite a paradoxical expansion of HSC-supportive niche cells, including osteoblasts, mesenchymal stem cells, and endothelial cells. Expression analysis showed that the Col(2.3)+/Rs1+ osteoblasts had decreased expression of key HSC-maintenance genes, including Sdf1, Vcam1, and Angpt1, accounting for the impaired ability to support HSC function. These findings indicate that long-term activation of Gs-GPCR signaling in osteoblasts of the HSC niche decreases HSC-supportive activity and leads to lineage-specific hematopoietic defects. Our results also suggest that long-term activation of Gs-GPCR signaling, such as in medical conditions of excess parathyroid hormone, may lead to

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adverse effects on endogenous HSCs and contribute to hematopoietic disorders such as anemia.

Poster Board Number: T-2118

AGE-SPECIFIC ALTERATIONS IN A NUMBER AND EFFICIENCY OF CLONING THE STROMAL CELLS-PRECURSORS OF HEMOPOIETIC AND LYMPHOID ORGANS FROM EXPERIMENTAL ANIMALS

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The analysis of specific differences in age-related alterations in the number of colony-forming cells-precursors of fibroblasts (CFU-F) in bone marrow, thymus, and spleen of CBA mice, guinea pigs, and Wistar rats, as well as the efficiency of their cloning (CE-F) was carried out with the use of mathematic method of gradient decline. In addition, those parameters were examined in SAMP (rapidly aging animals) and SAMR (with normal rate of aging) murine strains and in tests with the use of cross heterotopic transplants of bone marrow and spleen between the differently aged animals. The study revealed that reliable decrease in CFU-F number, the morphological change in their colonies being formed and the efficiency of stromal cells-precursors' cloning in all the animals tested were observed in the course of bone marrow, thymus and spleen aging. Most significant and rapid decrease in CFU-F amount was observed in guinea pig and mouse thymus namely by 75-, and 12-fold, respectively.The age-specific alteration in CFU-F quantity and their CE-F vary in the extent of expressiveness, age of the different strains of experimental animals and in animals of one strain in different organs that seems to depend on the physiological characteristics, peculiarities of body aging, life time of animals and functional role of the organs. Data obtained on the earlier age-specific decrease in the number of CFU-F in SAMP murine strain (9-12 mo.) as compared with the SAMR murine strain (16-19 mo.) and experiments with cross heterotransplants demonstrate that alongside with self age-related alterations in stromal tissue, the body as a whole influences it while aging. As far as it is well-known that CFU-F population in spleen and thymus involves the inducible osteogenic cells and bone marrow comprises determinate CFU-F, therefore the results obtained indicate the possibility of reducing the number of both categories of stromal precursors due to aging that could be considered as one of the reasons for age-related osteoporosis. This work was supported by RFBR grant 11-04-96037r_ural_a and administrative body of Perm Region.

Poster Board Number: T-2119

HOXB4 OVEREXPRESSION INCREASES PRIMITIVE CORD BLOOD CD34 POSITIVE HEMATOPOIETIC PROGENITORS CELLS

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Background: homeobox B4 (HOXB4) overexpression induces enhanced self-renewal of murine hematopoietic stem cells (HSCs) without compromising differentiation or homeostatic regulation of the HSC pool size. However, the effects of HOXB4 overexpression have not been evaluated so far for the expansion of human cord blood hematopoietic stem cells as well. We asked ourselves whether HOXB4 overexpression could induce primitive human cord blood HSCs or not. Material and Method: HSCs were transduced with lentiviral particle containing HOXB4. HOXB4 over expressing HSCs were *ex vivo* expanded for 8 days. Thereafter, expanded HSCs were evaluated both phenotypically by flow cytometry and functionally by clonogenic assay and LT-CIC. Results: HOXB4high HSCs were more enriched for CD34+ hematopoietic progenitor populations. Subsequently, clonogenic ability evaluation showed that the colony formation capacity of HOXB4high HSCs also significantly increased relative to control. Thereafter, the results of LT-CIC showed enrichment of primitive HSCs. Discussion: Altogether, our findings indicate that expansion of CD34+ hematopoietic progenitors were accompanied with self-renewal divisions of primitive subpopulation of HSCs. The expansion of primitive HSCs is the most important goal of *ex vivo* expansion of HSCs because of very high capacity of primitive HSCs in treatment of a wide range of hematologic diseases.

Poster Board Number: T-2120

EFFECT OF THE SURFACE DENSITY OF NANOSEGMENTS IMMOBILIZED ON CULTURE DISHES ON *EX VIVO* EXPANSION OF HEMATOPOIETIC STEM AND PROGENITOR CELLS FROM UMBILICAL CORD BLOOD

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Hematopoietic stem and progenitor cells (HSCs) are multipotent cells that have the specific capacity to self-renew and differentiate into all type of mature blood cells. Intravenous infusion of HSCs has been commonly performed to treat patients suffering from hematological disorders and malignant diseases after radiation and/or chemotherapy. Umbilical cord blood (UCB) is an attractive source of hematopoietic stem and progenitor cells (HSCs) for HSC transplantation. However, the low number of HSCs obtainable from a single donor of UCB limits direct transplantation of UCB to the treatment of pediatric patients. In this study, we investigated the *ex vivo* expansion of HSCs cultured on biomaterials grafted with several nanosegments, i.e., polyamine, fibronectin, RGDS, and CS1 (EILDVPST), at several surface densities. No systematic research has investigated the effect of nanosegment species such as FN, CS1, RGDS, and segments with amino groups immobilized on culture dishes on the *ex vivo* expansion of HSCs. Furthermore, no studies have examined the effect of the surface density of the nanosegments immobilized on culture dishes on the *ex vivo* expansion of HSCs. In this study, polymers with amino groups were grafted onto polystyrene dishes at various surface densities, and the effects of the surface densities of the amino groups on the *ex vivo* expansion of HSCs were investigated. Furthermore, polystyrene dishes with FN, CS1, CS1i, RGDS, RGES, or segments with amino groups were prepared systematically, and the optimal nano-segments for the *ex vivo* expansion of HSCs on the biomaterials were examined. This is the first study on the effect of the surface density of nanosegments immobilized on culture dishes on the *ex vivo* expansion of HSCs. An amount of grafted amino groups less than 0.80 residual μmol/cm2 on the dishes was effective for the *ex vivo* expansion of HSCs in 2D culture. A high amount of grafted amino groups on the dishes hindered the *ex vivo* expansion of HSCs. There was no direct correlation between fold expansion of HSCs and physical parameters of the culture dishes, i.e., RMS surface roughness and water contact angle, while HSCs cultured on dishes containing a

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high concentration of CS1 showed much higher fold expansion of HSCs and colony-forming units (CFU-GEMM) than those on other dishes, such as fibronectin-grafted and polyamine-grafted dishes. These data suggest that the specific interaction between HSCs and CS1 helps to maintain the pluripotency of HSCs during the *ex vivo* expansion of HSCs. CS1 is one of the cell-binding domains of fibronectin. However, the *ex vivo* expansion and CFU expansion of HSCs cultured on fibronectin-grafted dishes were less than those on PS-CS1-H dishes, although the *ex vivo* expansion and CFU expansion of HSCs on PS-FN dishes were higher than those on PS or TCPS. The presentation of specific binding site number of CS1 is 500 times (440,000/880) higher than that of fibronectin at the same grafting weight density of CS1 and fibronectin on dishes, because of low molecular weight of CS1 (approximately 880 dalton from 110 [average molecular weight of amino acids] x 8 [No. of amino acids in CS1]) than fibronectin (440,000 dalton). The presentation of specific cell-binding domains of CS1 at high concentrations seems to be important for the signal transduction that promotes the *ex vivo* expansion of HSCs.

Poster Board Number: T-2121

FROM HSC-CD34+ CELLS TO OSTEOCLAST IN A MODIFIED LTC ASSAY

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Osteoclasts (OCs) are multinucleated giant cells responsible for bone resorption and play an important role in normal skeletal remodeling. Recent studies suggest that osteoclasts are important component of stem cells niche and are involved in the regulation of haematopoiesis. Since several lines of evidence demonstrate that osteoclasts derive from haematopoietic CD34 cells, there is not clear information about the real osteoclast progenitor and its differentiation. In this study, to investigate osteoclastogenesis, we developed a 5 week 2-phase culture of human CD34 cells to induce mature osteoclastogenesis. At the same time we assessed the effect of a co-culture system of mouse stromal cell line MS-5 and CD34 cells on osteoclastogenesis. In the first 2 week, CD34 cells from PB were induced to proliferate and to commit to OC by co-culturing them on MS-5 cells in the presence of the following cytochines: SCF, FL, TPO and M-CSF. In the next phase (3 weeks), differentiation and maturation were induced by changing the combination of growth factors by adding: M-CSF, RANK-L. Differentiation was evaluated by TRAP staining, and RT-PCR which assessed the expression of c-fms, RANK, MMP-9. Resorption pit formation was also evaluated. After 5 weeks the output of cell population displayed the full range of OC differentiation markers. Stimulated CD34 generated functional OCs that formed extensive resorption lacunae on mineralized surface. To investigate if the OCs are derived from the monocyte-macrophage fusion, we analyzed the kinetics of OCs generation during culture (the CD34 expression and the acquisition of CD14, M-CSF and RANK-L) by flow cytometry and in-vitro imaging was performed during the 4°-5° weeks of the modified LTC assay. In this study, we show that MS-5 is able to induce osteoclastogenesis. Our system provides a new, robust, and simple methods for investigating the mechanism of osteoclast development (commitment and differentiation) from CD34 hematopoietic cells.

Poster Board Number: T-2122

MAINTENANCE AND EXPANSION OF HEMATOPOIETIC STEM CELLS THROUGH REGULATION OF WNT AND MTOR PATHWAYS

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Hematopoietic stem cells (HSCs) are able to self-renew and differentiate into all blood cell lineages. The signaling pathways that regulate HSC self-renewal and differentiation are not well understood. In the bone marrow, HSC self-renewal and lineage commitment also depend on complex interactions with the microenvironment. Recent evidence suggests that HSCs reside in a low perfusion, reduced nutrient niche and that nutrient sensing pathways contribute to HSC homeostasis. Our published work demonstrated that GSK3 plays critical roles in HSC homeostasis and is involved in the Wnt/ß-catenin and mTOR pathways. Here we report that suppression of the mammalian target of rapamycin (mTOR) pathway, an established nutrient sensor, combined with activation of canonical Wnt/ß-catenin signaling, allows the *ex vivo* maintenance of human and mouse long-term HSCs under cytokine-free conditions. We also show that combining two clinically approved medications that activate Wnt/ß-catenin signaling and inhibit mTOR lead to expansion of long-term HSCs in vivo.

Poster Board Number: T-2123

MIR 92A REGULATES THE DIFFERENTIATION OF CD34+ CELLS VIA CONTROLLING CIRCADIAN TARGET GENES

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The endogenous circadian system via a set of autoregulatory clock genes drives the rhythm of many physiological and behavioral processes. Abnormal expression of clock genes is implicated in many pathologic disorders like metabolic syndrome, obesity, premature aging and abnormal sleep cycle. Previously, we reported that the defects in clock gene expression of endothelial progenitor cells (EPCs) from Type II diabetic rats are involved in development of diabetic retinopathy. In the present study, we hypothesized that the differentiation of EPCs towards endothelial cells is under the control of clock genes and that in diabetes this pattern is altered. $CD34^+$ cells (~95% starting purity) obtained from healthy or diabetic donors were isolated by flow cytometry. $CD34^+$ cells were placed in either hematopoietic supporting media (Stem cell factor, IL-3, IL-6, Flt1) or endothelial differentiation media (VEGF, Serum and other growth factors) and 1) evaluated at 0 hr and 96 hr using a combination of CD133 and CD144 markers by flow cytometry; 2) sampled every 4 hr for 4 days to evaluate the expression pattern of the clock genes (*Clock*, *Bmal1*, *Per1*, *Per2*, *Cry1* and *Cry2*) using qRT-PCR. Using microarrays, the miRNA signature of differentiating $CD34^+$ cells was compared to early EPCs and outgrowth endothelial cells (OECs). Data was analyzed using Ingenuity pathway analysis software. $CD34^+$ cells under hematopoietic supporting media rapidly began expressing the stem cell marker, CD133 ($p<0.05$) with a significant decrease in expression of the endothelial marker CD144 ($p<0.05$) while exposure to the endothelial supporting media showed a reverse trend with a dramatic increase ($p<0.05$) in CD144 expression. The mRNA pattern of clock genes, in $CD34^+$ cells sampled every 4

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hr showed an increase in amplitude of *Bmal1*, *Per1*, *Per2*, *Cry1* and *Cry2* of oscillations upon differentiation while expression of *Clock* remained suppressed. Using specific siRNA to *Per2* (2 fold; $p<0.05$) and *Cry2* (1.5 fold; $p<0.05$) we observed a reduction in CD133 expression under hematopoietic supporting conditions. *Per2* and *Cry2* mRNA showed a complete suppression in OECs, the most differentiated population of the endothelial progenitor cells examined. Microarray analysis mapped 59 miRNAs in non-diabetic and 56 miRNAs in diabetic EPCs regulating 1372 mRNAs among the three different populations of EPCs. Further analysis of miRNA target filter revealed unique 25 miRNAs targeting clock genes. qRT-PCR to confirm miRNA targets identified in microarray analysis showed a 50% reduction ($p<0.05$) in the expression of miR92a in diabetic $CD34^+$ cells. In conclusion, clock genes play a pivotal role in differentiation of $CD34^+$ cells and correcting the period and amplitude of clock gene oscillations in dysfunctional $CD34^+$ cells may impact their reparative function in disease states such as type 2 diabetes. Interventional approach restoring proper miRNA regulation paves a new therapeutic option for optimizing progenitor cell function prior to their use in autologous cell therapy.

Poster Board Number: T-2124

TISSUE CHIMAERISM AFTER BONE MARROW CELL TRANSPLANTATION.

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Bone marrow (BM) cell transplantation represents a life-saving treatment for syndromes involving BM failure and thalassemia. To trace distribution of transplanted cells in the recipient organism, we utilized transgenic eGFP mice as donors whose cells were tagged to endogenous vectors. BM ablation in recipient C57Bl6/J mice was induced by whole body lethal irradiation 9.5 Gy that severely damages haemopoiesis. Irradiated splenectomized or non-splenectomized mice were rescued by i.v. transplantation of $5x10^6$ bone marrow cells performed 3 hours following irradiation. Recipient tissues were examined by independent methods (incl. quantitative PCR, flow cytometry, immunohistochemistry and fluorescent microscopy) to determine the rate of colonisation of tissues in non-splenectomised and splenectomised mice by transplanted cells at different survival periods (8 to 180 days). A successful transplantation led to establishment of a stable donor-cell chimaerism in BM (approx. 80%) and in peripheral blood. Initial low engraftment in the thymus led to 100% chimaerism after a 180-day survival in non-splenectomised mice whereas in the small intestine and liver the highest levels were reached by day 180 in splenectomised mice (21% and 14% respectively). Results obtained from histological examination correlated with data obtained from qPCR analysis and provided detailed information on changes in local distribution of transplanted cells. In the small intestine, the highest positivity was observed in the mucosa while the epithelial lining contained only sporadic GFP^+ intraepithelial lymphocytes. Stroma of in intestinal villi contained up to 43% GFP^+ cell because of a high turnover and production of new connective tissue cells. Inside the liver parenchyma, cell chimaerism reached 26% after 180 days; GFP^+ cells participated in a turnover of stromal elements incl. endothelial and Ito cell while hepatocytes remained GFP-negative. BM cells after systemic i.v. administration do not necessarily engraft homotopically back to BM. Our data demonstrate BM transplantation induces a stable post-transplantation chimaerism in several recipient tissues.

Poster Board Number: T-2125

ZINC-FINGER NUCLEASE MEDIATED CORRECTION OF ALPHA-THALASSEMIA IN IPS

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iPS technology has the potential to provides an unlimited source of cells for regenerative medicine. Since the first seminal report in 2006, significant progress has been made toward the development of practical applications. For instance, several groups have developed transgene-free reprogramming methods. In the hematopoietic system, one major application would be a cure for the hemoglobinopathies. Recent reports have demonstrated insertion of lentivirus carrying a therapeutic beta-globin transgene in iPS from beta-thalassemia patients, and correction of the mutation causing sickle cell anemia using homologous recombination. The gene corrections were demonstrated at the genetic level and evidence was provided that the correction was functional at the protein or mRNA levels by differentiating the iPS into erythroid cells. however, levels of expression were very low because current iPS differentiation protocols yield erythroid cells that express mostly embryonic and fetal hemoglobin and only trace amounts of beta-globin. We report here correction of alpha-thalassemia major hydrops fetalis using zinc finger nuclease mediated insertion of therapeutic globin transgenes in the AAVS1 sites within the PPP1R12C gene on human chromosome 19. Four types of constructs were tested. With the best one, which contains the beta-globin miniLCR driving the alpha-globin promoter and the alpha-globin gene with its 2 introns, homozygous insertion in the two copies of the PPP1R12C genes led to complete correction of chain imbalance in erythroid cells differentiated from the corrected iPS (alpha-like/beta-like ratio = 1 and 0.9 respectively in basophilic and orthochromatic erythroblasts). Because the alpha-globin genes are expressed at very high level in cells derived from iPS, globin chain measurement could be made precisely by HPLC in cells where the globin gene are expressed at their maximum level. ZFN-mediated site specific insertion has very attractive characteristics for gene therapy in iPS. Firstly, the risk of insertional mutagenesis is eliminated provided that a "safe harbor" is identified. Secondly, and equally importantly, the use of this technology should eventually simplify clinical implementation because a single set of validated constructs and reagents could be used to correct the large variety of mutations that cause the hemoglobinopathies. This presents considerable practical and economic advantages over having to design and validate custom constructs for each particular mutation. To determine if AAVS1 is a safe harbor, we analyzed expression of the 10 closest genes to the site of integration. We found that transgene insertion at AAVS1 had no effects on neighboring genes in undifferentiated cells but that it activated four genes in erythroid cells derived from iPS. Because the level of activation were relatively small, it is unclear if that disqualifies the site as a safe harbor. Nevertheless, these results suggest that additional landing pad should be developed and tested. The major conclusion of the study is that ZFN-mediated gene insertion is a very effective method to obtain high-level, quasi normal expression of globin therapeutic cassettes.

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Poster Board Number: T-2126

HISTONE ACETYLTRANSFERASE MORF AND MOZ ARE ESSENTIAL FOR HEMATOPOIESIS AND SELF RENEWAL OF HEMATOPOIETIC STEM CELLS

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Myst-type histone acetyltransferases, MOZ (MOnocytic leukemia Zinc finger; Myst3, KAT6A) and its related MORF (MOz Related Factor; Myst4, KAT6B), are involved in chromosome translocations associated with FAB-M4/5 subtypes of acute myeloid leukemia. We have reported that MOZ is essential for hematopoietic cell development and self-renewal of hematopoietic stem cells. To explore the possibility MORF also plays important roles in hematopoiesis, we generated Morf-deficient mice with homologous recombination methods. Morf-/- mice were smaller than their wild-type littermates and died within 4 weeks after birth on C57BL/6 background. In Morf-/- fetal liver, Flt3-negative KSL (c-Kit+, Sca-1+, Lineage-) cells containing hematopoietic stem cells were slightly decreased. Defects in megakaryopoiesis were also observed. Transplantation analysis showed that Morf-/- fetal liver cells less efficiently reconstituted hematopoiesis when compare to wild-type cells. Especially, contribution of Morf-/- cells to KSLs and myeloid compartments were decreased in recipient mice. To reveal relationship between MORF and MOZ in hematopoiesis, we generated double heterozygous (Moz+/-, Morf+/-) mouse. Double heterozygous mice were smaller than wild-type littermates and died at least 4 weeks after birth. Number of KSL cells, especially Flt3- KSL cells and common myeloid progenitors were decreased in the double heterozygous embryos. The double heterozygous fetal liver cells also displayed less activity to reconstitute hematopoiesis than Moz+/- or Morf+/- cells. Since Morf-/- mice were alive at adult on a mixed C57BL/6/DBA2 genetic background, we investigated adult hematopoiesis in these mice. Morf-/- mice were smaller than their wild-type littermates and had small number of thymocytes and splenocytes. In Morf-/- bone marrow, CD34-negative KSLs including hematopoietic stem cells were decreased. Aged or 5-FU treated Morf-/- mice had reduced number of hematopoietic stem cells. MORF as well as MOZ interacts with AML1 and PU.1 that were important for hematopoiesis and stimulated transcription of their target genes. These results suggest that MORF and MOZ play important roles in hematopoietic lineage cell development and self-renewal of hematopoietic stem cells, and that they modulate AML1 or PU.1-dependent transcription.

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THE ROLE OF GH/IGF-I DEFICIENCY IN STEM CELL PROTECTION, RENEWAL AND HEMATOPOIETIC REGENERATION

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The stem-cell aging hypothesis postulates that aging is caused, in part, by the loss of functional adult stem cells required for tissue regeneration and repair. The mechanisms regulating stem cell stress resistance and its contribution to age-related degenerative changes, however, remain unclear. Understanding cellular signaling that can protect the stem cells from chronic toxicity and from being depleted or exhausted after constantly replenishing damaged tissues may shed a light on stem-cell aging hypothesis and augment existing chemotherapy and stem-cell transplant therapies. Dampening of IGF-I signaling, via either dietary intervention or genetic modification, is known to promote longevity in organisms ranging from yeast to mammals. In this study, we reported the potential anti-aging and/or rejuvenation effect of low circulating IGF-1 in the murine hematopoietic system. As in aging, multiple-cycles of chemotherapy-induced chronic oxidative stress caused long-term stem cells/progenitors impairment, in addition to the immediate tissue damages. We hypothesize that GH/IGF-I deficiency can also benefit the hematopoietic system in overcoming the cumulative long-term toxicity associated with chemotherapy. Mice with either regular or manipulated low IGF-I levels were challenged with multiple-cycle cyclophosphamide (CP) treatments for 4-6 months and their hematopoietic system was examined. Preliminary results suggest improved hematopoietic protection or recovery possibly supported by the enhanced long-term repopulation capacity of the protected hematopoietic stem cell/progenitors in mice with reduced circulating IGF-I. Interestingly, a newly discovered type of pluripotent stem cells, Very-small-embryonic-like (VSEL)(Oct-4+ SSEA-1+Sca-1+Lin-CD45-) stem cells, was also found to respond to the changes of IGF-I level. VSEL stem cells are known play an important role in repair of various tissues. Whether the low-IGF-I recruited VSEL directly supply the HSCs pool or indirectly repair the stem cell niche and eventually enhance hematopoietic regeneration, however needs to be further investigated. The protective effect of low IGF-I was then confirmed with human amniotic fluid stem cells (hAFSC), as an *in vitro* model, to investigate the therapeutic potential of manipulating IGF-I signaling in human stem cells. Here we present a study linking stem cell aging with stem cell exhaustion under chronic toxicity of chemotherapy and demonstrate that reducing IGF-I signaling may augment cellular stress response and therefore improved hematopoietic recovery from long-term chemotoxicity.

Poster Board Number: T-2128

REPROGRAMMING OF COMMITTED ERYTHROID PROGENITORS TO MAGAKARYOCYTES BY FLI1 AND ERG1 TRANSCRIPTION FACTORS

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Megakaryocytes and erythrocytes are derived from common progenitors called megakaryocytic-erythroid progenitor cells (MEPs). However, megakaryocyte lineages follow a very different gene expression program and morphological transformation from that of erythrocyte lineages. Although, previous studies suggest that transcription factors ERG1 and FLI1 might play roles in directing MEP differentiation toward megakaryocyte lineage, the functions of these transcription factors in directing and maintaining megakaryocyte gene expression program are still not fully elucidated. To study the roles of ERG1 and FLI1 in directing and maintaining megakaryocyte gene expression program, we overexpressed Erg1 and Fli1 genes in purified human erythroid progenitor cells (which are derived from bone marrow and umbilical cord blood mono-

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nuclear cells by magnetic activated cell sorting with the use of erythrocyte lineage markers, anti-CD71 and anti-glycophorin A antibodies). Phenotypic characteristics of the transformed erythroid cells were subsequently determined by cell morphology and the expression profiles of several erythroid and megakaryocyte lineage markers as well as their ability to form megakaryocyte colonies under appropriate culture condition. Our results showed that the percentages of Erg1- and Fli1-overexpressed erythroid cells which express megakaryocyte-lineage marker CD41 was 27-folds higher than those of non-transformed erythroid cells (35.7 ± 2.8 % vs. 1.3 ± 0.6 %, P<0.05) after 5 days of culture in megakaryocyte inducing medium. In addition, the morphology of the overexpressed Fli1 and Erg1 erythroid cells was change from small cells with high nuclear/cytoplasmic ratio (which are typical for erythroid) to large multi-nucleated cells that expressed megakaryocyte specific marker, glycoprotein IIb/IIIa while the morphology of non-transformed erythroid cells were remain the same. Moreover, the transformed erythroid cells could also generate several megakaryocyte colony-forming units under collagen-based megakaryocyte culture condition while non-transformed erythroid could not generate any megakaryocyte colonies under the same condition. In conclusion, our study indicates that both FLI1 and ERG1 play critical roles in directing MEP differentiation toward megakaryocyte lineage. Moreover, the expressions of Fli1 and Erg1 genes are sufficient to reprogrammed committed erythroid cells to megakaryocytes as determined by morphology, the expression profile of megakaryocyte specific marker and their ability to form megakaryocyte colonies under appropriate culture condition. The function of these reprogrammed erythroid cells is currently investigated.

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SAFETY AND SEIZURE CONTROL IN PATIENTS WITH MEDICALLY REFRACTORY MESIAL TEMPORAL LOBE EPILEPSY TRANSPLANTED WITH AUTOLOGOUS BONE MARROW MONONUCLEAR CELLS

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Temporal lobe epilepsy (TLE) is a highly prevalent syndrome in patients with seizures and it is usually refractory to drug treatment. Anatomical and physiological changes are often present in patients with TLE such as mesial temporal sclerosis (MTS) characterized by extensive cell loss, reactive gliosis, mossy fiber sprouting and neurogenesis in the hippocampus and para-hippocampal structures. Hippocampal sclerosis can be verified by magnetic resonance imaging (MRI). Stem cells are proven to have the ability to reduce seizure frequency and to preserve the hippocampal neuronal networks in experimental model of chronic epilepsy. The aim of this study is to verify the safety and the therapeutic potential of bone marrow mononuclear cells (BMMC) transplantation on seizure control in patients with medically refractory mesial temporal lobe epilepsy (MTLE). To be eligible, patients had to be at least 18 years old and have to be diagnosed as MTLE according the Commission report of the International League Against Epilepsy. They were submitted to neurological evaluation, MRI study with hippocampal volumetry, 72 hour- VideoEEG for epileptic seizure recording and laboratory tests. Also, patients were evaluated with a standardized neuropsychological test battery, focusing on verbal and nonverbal memory domains, consisting of the Wechsler Memory Scale-Revised, the Rey Auditory Verbal Learning Test, and the Rey Visual Design Learning Also, patients were evaluated with a standardized neuropsychological test battery, focusing on verbal and nonverbal memory domains, consisting of the Wechsler Memory Scale-Revised, the Rey Auditory Verbal Learning Test, and the Rey Visual Design Learning Test. All patients who fulfilled selection criteria were invited to take part. After informed consent those with seizures originating in one temporal lobe that had consistent data from MRI and neuropsychological tests showing convergence for the same side, underwent BMMC transplant by selective posterior cerebral artery catheterism. This study was approved by Ethics Committee from our institution and National Committee on Ethics in Research and it is registered on www.clinicaltrials.gov (NCT00919266). All patients who fulfilled selection criteria were invited to take part. After informed consent those with seizures originating in one temporal lobe that had consistent data from MRI and neuropsychological tests showing convergence for the same side, underwent BMMC transplant by selective posterior cerebral artery catheterism. This study was approved by Ethics Committee from our institution and National Committee on Ethics in Research and it is registered on www.clinicaltrials.gov (NCT00919266). Twenty patients have already undergone this procedure. Fourteen patients were submitted to 3 and 6 months follow-up. After a follow-up of 6 months, nine patients (64.3%) were free of disabling seizures (Engel's Class I). There were no complications related to the procedure. No additional lesions were detected on MRI. Also, no significant hippocampal volumetric change was observed. The EEG analysis showed a decrease of theta activity and spike density. Given these data we believe that BMMC autologous transplant for the treatment of MTLE is indeed feasible and seems to be safe so far. Seizure control achieved in this first experience gives us a very promising path in therapeutic potential of stem cells transplant in this population.

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NERVE GROWTH FACTOR IN COMBINATION WITH COLLAGEN HAS NOVEL ACTIVITIES IN PRESERVING GROWTH FACTOR-ACTIVATED HEMATOPOIETIC STEM CELL SURVIVAL AND EXPANSION *IN VITRO*

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Background: Many conditions have been found to stimulate the expansion of cells with long term hematopoietic repopulating ability, but conditions that preserve durable self-renewal remain elusive. We have previously shown that hematopoietic stem cells (HSC) from adult mouse bone marrow stimulated to divide in single-cell serum-free cultures execute a limited number of self-renewal divisions in the presence of 300 ng/mL Steel Factor + 20 ng/mL IL-11. However, only 40% of cells in highly purified HSC suspensions survive and form clones, and the yield of HSCs with durable self-renewal activity after one week is <10%. Medium conditioned by murine UG-26 fibroblasts (CM) improves both of these endpoints. It was therefore of interest to identify the responsible factors. Methods: Affymetrix array analysis was performed on RNA obtained from highly purified HSCs (CD45+EPCR+CD150+CD48-, "E-SLAM" cells, ~ 50% HSCs) before and after their incubation for 6 hours in CM only, in CM+100 ng/mL SF+20 ng/mL IL-11 (+GFs), or +GFs only. We then used the GO classifier to identify "receptor activity" transcripts in fresh ESLAM cells and "extracellular region" transcripts in UG26 cells (data online at GSE11589) to identify factors secreted

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by UG26 cells that had a putative receptor on ESLAM cells. Evidence of differential pathway activation in the presence of factors in CM was obtained by comparing ESLAM cells stimulated ±CM (±GFs). For clonogenic assays, single ESLAM cells were sorted into each well of a 96-well plate +GFs and one or more candidate CM factors. Input cells were then scored for their ability to generate >=10 cells after >=1 week. HSCs in 7-day cultures initiated with 30 ESLAM cells were quantified in limiting dilution transplant assays using congenic, sublethally irradiated W41/W41 recipients (3 dilutions/experiment) and detection of positive mice 16 weeks later (mice with >1% donor-derived circulating WBCs and a >=1% donor contribution to the myeloid WBCs). Results: We identified transcripts for 172 "extracellular region" proteins in the UG26 cells for which there were also matching transcripts in the fresh ESLAM cells for one or more interacting proteins. Of the 430 tested Reactome pathways surveyed, we found 250 for which some members showed significantly altered transcript expression ($p<0.05$) between the tested conditions. Within this subset were a number of pathways for which a cognate cytokine gene was expressed in the UG26 cells. These included "signalling by NGF" and a "collagen-mediated activation cascade". From these data, a number of factors were selected and clonogenic assays used to identify those that promoted ESLAM cell survival and clone formation in cultures +GFs. Of the factors tested, we found collagen, NGF (nerve growth factor), and Matrigel increased ESLAM cell cloning efficiency to 72%, 86%, and 100%, respectively (holm-adjusted p values <=0.01). Of the defined factors, NGF + collagen gave the highest clonogenicity (97%). Transplants showed that 7-day cultures+GFs that also contained NGF+collagen maintained HSCs numbers at 74% of input levels (95% CI 0.37-1.49) with retention of multi-potency and subsequent *in vivo* self-renewal activity. Conclusions: NGF+collagen, 2 factors produced by stromal cells, in addition to SF+IL-11 promote the survival and proliferation of HSCs in vitro with significantly enhanced preservation of HSC functional properties.

Poster Board Number: T-2131

PYRUVATE KINASE M2 PLAYS DISTINCT ROLES IN HEMATOPOIETIC STEM CELLS AND LEUKEMIA

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It has been increasingly realized that different cell states employ different metabolic modes. Whether the metabolic mode is a determinant of cell state is unclear, but studies in cancer cells suggest that metabolic shifting from glycolysis toward oxidative phosphorylation can modify in vitro and in vivo function of tumorogenic cells. Cancer cells uptake glucose at a rate higher than normal cells but convert the majority of glucose into lactate even in the presence of oxygen, a phenomenon known as aerobic glycolysis or the Warburg effect. Normal somatic cells thought to also preferentially use anaerobic metabolism are tissue stem cells, particularly the self-renewing hematopoietic stem cells (HSC) resident in the hypoxic microenvironment of the bone marrow. Recently the molecular basis for the Warburg effect in cancer cells has been identified as due to a specific isoform of pyruvate kinase M2 (PKM2). In contrast, normal tissues that use oxidative phosphoryation as their major metabolic mode express the alternatively spliced isoform PKM1. Interestingly, we observed that cells in the hematopoietic lineage, including HSC, predominantly express PKM2. In order to understand the role of PKM2 and glycolysis in normal hematopoiesis as well as in hematological malignancy, we have generated a genetically modified mouse strain that allows conditional deletion of the PKM2 specific exon. Our data show that PKM2 deletion does not compromise normal HSC function under homeostatic conditions. Rather, loss of PKM2 results in a competitive advantage over wild-type HSC, as evidenced by enhanced bone marrow reconstitution in a competitive transplantation assay. The altered cellular function by PKM2 deletion is accompanied with decreased glycolysis and increased oxidative phosphorylation specifically in the primitive stem/progenitor cell population. Notably, however, the absence of PKM2 appears to markedly disadvantage the establishment of leukemia in mice when hematopoietic cells express human leukemogenic alleles associated with either acute or chronic myeloid leukemia. Such a distinction in response between normal and malignant cells is unprecedented to our knowledge and may offer a highly unique opportunity to advantage normal while disadvantaging malignant cells of the hematopoietic lineage by targeting a single metabolic enzyme.

Poster Board Number: T-2132

GNAT-LIKE PROTEIN DOWN-REGULATES GLOBIN SYNTHESIS BY BINDING ACETYL COENZYME A

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Globin synthesis is crucial event in erythroid differentiation and regulated by hisotne acetylation. We identified novel molecule that regulates histone acetylation through our database of anemic zebrafish induced by cold-exposure. This molecule belongs to the family of GCN5-related N-acetyltransferase (GNAT), which functions in histone acetylation. Mouse *GNAT-like* gene was up-regulated as hematopoietic stem cells differentiate into mature erythrocytes synchronizing with increase of *globin* gene expression. Although this GNAT-like protein could bind to acetyl coenzyme A (acetyl CoA), it lacked histone acetylation activity. Ectopic expression of mouse *GNAT-like* gene in erythroleukemia cells decreased histone acetylation and *globin* gene expression, whereas knock down of *GNAT-like* gene in zebrafish embryos increased gene and protein expressions of globin. Taken together, this GNAT-like protein down-regulates globin gene synthesis likely through competing with other GNATs for acetyl CoA binding.

Poster Board Number: T-2133

TRANSCRIPTOME ANALYSIS WITHIN THE IMMGEN DATABASE IDENTIFIES REGULATORS OF PROGENITORS AND STEM CELLS.

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Although hematopoietic stem cells are the best characterized of adult tissue-specific stem cells, surprisingly little is known about the mechanisms that regulate their central properties of self-renewal and multi-lineage potential. Towards improving the molecular understanding of HSCs and their proximal multi-potent and oligo-potent progenitor subsets, we performed expression profiling of these primitive progenitors and analyzed the data within the context of the ImmGen microarray database comprised of over 700

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microarrays that encompasses the vast majority of immune cell types. Principle component analysis positioned all hematopoietic stem and progenitor subsets (HSPCs) in a cluster at a central position in relation to downstream effector cells showing the relatedness of HSPC subsets and consistent with their role as undifferentiated precursors of all blood lineages. Gene set enrichment analysis revealed that HSPCs were most enriched for cell growth and cell cycle pathways in line with their role as transit amplifying cells. Analysis of the multipotent subsets (MP) identified a novel group of genes encoding KRAB-domain containing proteins, predicted to be transcriptional repressors- suggestive of a role in suppressing lineage commitment in these otherwise uncommitted cells. Genes involved in transcriptional regulation were identified in HSCs and co-expression analysis of these factors showed that those with established roles in HSC quiescence and self-renewal clustered separately from a set of factors of unknown function in HSCs. Finally, enforced expression of one transcription factor, Hlf was able to imbue stem and progenitors with a sustained primitive immune-phenotype, and extensive self-renewal and multi-myeloid lineage potential. This work, within the ImmGen project, presents a reliable reference of whole-transcriptome data across the immune system, finding novel genes as candidate regulators of adult HSCs.

Poster Board Number: T-2134

DISRUPTION OF TET2 LEADS TO ENHANCED SELF-RENEWAL AND COMPETITIVE REPOPULATING CAPACITY OF FETAL LIVER HEMATOPOIETIC STEM CELLS

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TET2 (*ten-eleven-translocation 2*) gene has been reported to be frequently mutated in various human myeloid and lymphoid malignancies. These observations suggest critical roles of *TET2* dysfunction in molecular pathogenesis of hematologic malignancies. Recent studies using conditional knockout mouse model indicated that mouse *Tet2* loss leads to clonal dominance of adult hematopoietic stem cells (HSCs) in competitive repopulation assay. However, self-renewal capacity of adult HSCs has never been addressed precisely by serial transplantation assay. In addition, the effect of *Tet2* loss on HSCs was never examined in the fetal livers (FLs). Since FL HSCs and adult HSCs differ in several aspects of their phenotypes and functions, we speculated that *Tet2* might be involved differently in the regulation of FL and adult hematopoiesis. To address this issue, we analyzed E14.5 FL cells from *Tet2* gene-trap (*Tet2*^{gt}) mice. RT-PCR analysis showed that over 99% of *Tet2* mRNAs from endogenous promoter were trapped by the gene-trap cassette in *Tet2*$^{gt/gt}$ mice, showing that Tet2^{gt} allele can be considered as a null allele. Initial analysis showed that *Tet2*$^{gt/gt}$ embryos developed normally, but most *Tet2*$^{gt/gt}$ mice were perinatally lethal. Interestingly, *Tet2*$^{gt/gt}$ embryos displayed significant increase in lineage (Lin)$^-$Sca-1$^+$c-Kit$^+$ (LSK) fraction compared to wild type (WT) (*Tet2*$^{+/+}$) littermate (1.45±0.62% vs. 0.85±0.34%, p=0.0027). In addition, common myeloid progenitor (CMP) fraction (IL7Rα$^-$ Lin$^-$ Sca-1$^-$ c-Kit$^+$ CD34$^+$ FcgRII/III^{low}) was significantly increased in *Tet2*$^{gt/gt}$ FLs compared to WT (9.04±1.09% vs. 6.26±0.53%, p=0.008). In serial transplantation assays, donor cells derived from *Tet2*$^{+/gt}$ and *Tet2*$^{gt/gt}$ FLs showed significantly higher peripheral blood chimerism in secondary and tertiary recipient mice as compared to that of WT cells, showing that disruption of *Tet2* leads to the enhanced self-renewal capacity of FL HSCs. Moreover, donor-derived HSC fraction (CD34$^-$LSK cells) was significantly expanded in the recipients of *Tet2*$^{gt/gt}$ FL cells, suggesting that increased self-renewal capacity is cell intrinsic to *Tet2*$^{gt/gt}$ HSCs. We have also examined differentiation of *Tet2*-mutant FL cells in the recipients' peripheral blood, and found that *Tet2*$^{gt/gt}$ cells displayed impaired differentiation to Gr-1$^+$CD11b$^+$ mature granulocytes. Liquid culture of FL cells with cocktails of cytokines *in vitro* demonstrated that *Tet2*$^{gt/gt}$ FL cells retained higher percentage and number of LSK, Lin$^-$ and c-Kit$^+$ cells after the culture for 7-days compared to WT cells, showing enhanced resistance of *Tet2*$^{gt/gt}$ cells to differentiative stimuli in *in vitro* culture. It is of note that *Tet2*$^{+/gt}$ mice showed a significant increase in LSK compartment in the BM compared to wild type littermate (0.48±0.11% vs. 0.32±0.04%, p=0.04). However, they presented no signs of extramedullary hematopoiesis such as splenomegaly and expansion of LSK cells in spleens during an observation up to 35-weeks.Taken together, we demonstrate that *Tet2* critically regulates self-renewal and long-term repopulating capacity of FL HSCs and has pleiotropic functions in hematopoietic differentiation. In addition, enhanced HSC self-renewal, expansion of HPC and myeloid progenitors and perturbed differentiation induced by *TET2* ablation likely to set molecular basis for malignant transformation, which explains high incidence of loss-of-function mutations of *TET2* in hematologic malignancies.

Poster Board Number: T-2135

DECOUPLING OF TUMOR-INITIATING ACTIVITY FROM STABLE IMMUNOPHENOTYPE IN HOXA9-MEIS1 DRIVEN AML

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Increasing evidence suggests tumors are maintained by cancer stem cells; however, their nature remains controversial. In a HoxA9-Meis1 (H9M) model of acute myeloid leukemia (AML), we found that tumor-initiating activity existed in three, immunophenotypically distinct compartments, corresponding to disparate lineages on the normal hematopoietic hierarchy_stem/progenitor cells (Lin$^-$kit$^+$), and committed progenitors of the myeloid (Gr1$^+$kit$^+$) and lymphoid lineages (Lym$^+$kit$^+$). These distinct tumor-initiating cells (TIC) clonally recapitulated the immunophenotypic spectrum of the original tumor *in vivo* (including cells with a less-differentiated immunophenotype) and shared signaling networks, such that *in vivo* pharmacologic targeting of conserved TIC survival pathways (DNA methyltransferase and MEK phosphorylation) significantly increased survival. Collectively, H9M AML is organized as an atypical hierarchy that defies the strict lineage marker boundaries and unidirectional differentiation of normal hematopoiesis. Moreover, this suggests that in certain malignancies tumor-initiation activity (or "cancer-stemness") can represent a cellular state that can exithat exists independently of distinct immunophenotypic definition.

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TIM-3 EXPRESSION IN THE VARIOUS TYPES OF HEMATOLOGICAL MALIGANANT LEUKEMIC STEM CELLS

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Acute myeloid leukemia (AML) originates from self-renewing leukemic stem cells (LSCs), an ultimate therapeutic target for AML. We identified T-cell immunoglobulin mucin-3 (TIM-3), as a surface

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molecule expressed on LSCs in most types of AML except for acute promyelocytic leukemia, but not on normal hematopoietic stem cells (HSCs). TIM-3+ but not TIM-3- AML cells reconstituted human AML in immunodeficient mice, suggesting that the TIM-3+ population contains most, if not all, of functional LSCs. We established an anti-human TIM-3 mouse IgG2a antibody having complement-dependent and antibody-dependent cellular cytotoxic activities. This antibody did not harm reconstitution of normal human HSCs, but blocked engraftment of AML after xenotransplantation. Furthermore, when it is administered into mice grafted with human AML, this treatment dramatically diminished their leukemic burden, and eliminated LSCs capable of reconstituting human AML in secondary recipients (Kikushige et al, Cell Stem Cell, 2010). We extended the analysis of TIM-3 expression in the various types of human hematological malignancies, and found that human TIM-3 was expressed in the vast majority of CD34+CD38- leukemic stem cell fractions of human myeloid malignancies including chronic myeloid leukemia, chronic myelononocytic leukemia and myelodysplastic syndrome (MDS). Especially in MDS, the expression level of TIM-3 on CD34+CD38- leukemic stem cells is dramatically up-regulated along with disease progression to AML, suggesting the important role of TIM-3 during leukemogenesis. We also intensively investigated the function of TIM-3 signaling in human hematological malignancies. In this presentation, we would like to discuss the further details of human TIM-3 function in myeloid leukemic stem cells.

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EFFECT OF NANOSEGMENTS ON *EX VIVO* EXPANSION OF HEMATOPOIETIC STEM AND PROGENITOR CELLS CULTURED ON SURFACE HAVING SEVERAL NANOSEGMENTS

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Umbilical cord blood (UCB) is an attractive source of hematopoietic stem and progenitor cells (HSPCs) for HSPC transplantation. However, the low number of HSPCs obtainable from a single donor of UCB limits direct transplantation of UCB to the treatment of pediatric patients. In this study, we investigated the *ex vivo* expansion of HSPCs cultured on biomaterials grafted with several nanosegments, i.e., polyamine, fibronectin, RGDS, and CS1 (EILDVPST), at several surface densities. No direct correlation was found between fold expansion of HSPCs and physical parameters of the culture dishes, i.e., surface roughness and water contact angle of the culture dishes. However, a small amount of grafted amino groups, less than 0.8 residual mol/cm^2, on the dishes was effective for the *ex vivo* expansion of HSPCs. A high amount of grafted amino groups hindered the *ex vivo* expansion of HSPCs on the dishes. HSPCs cultured on dishes with a high concentration of CS1 (2.40 residual mol/cm^2) showed greater expansion of HSPCs and more pluripotent colony-forming units (i.e., colony-forming unit-granulocyte, erythroid, macrophage, and megakaryocyte (CFU-GEMM)) than those on fibronectin-grafted and polyamine-grafted dishes. These data suggest that the specific interaction between HSPCs and CS1 helps to maintain the pluripotency of HSPCs during the *ex vivo* expansion of HSPCs. This is the first study on the effect of the surface density of nanosegments immobilized on culture dishes on the *ex vivo* expansion of HSPCs. An amount of grafted amino groups less than 0.80 residual mol/cm^2 on the dishes was effective for the *ex vivo* expansion of HSPCs in 2D culture. A high amount of grafted amino groups on the dishes hindered the *ex vivo* expansion of HSPCs. There was no direct correlation between fold expansion of HSPCs and physical parameters of the culture dishes, i.e., RMS surface roughness and water contact angle, while HSPCs cultured on dishes containing a high concentration of CS1 showed much higher fold expansion of HSPCs and colony-forming units (CFU-GEMM) than those on other dishes, such as fibronectin-grafted and polyamine-grafted dishes. These data suggest that the specific interaction between HSPCs and CS1 helps to maintain the pluripotency of HSPCs during the *ex vivo* expansion of HSPCs. CS1 is one of the cell-binding domains of fibronectin. However, the *ex vivo* expansion and CFU expansion of HSPCs cultured on fibronectin (FN)-grafted dishes were less than those on PS-CS1-H (CS1-grafted polystyrene [PS] with high concentration of CS1) dishes, although the *ex vivo* expansion and CFU expansion of HSPCs on PS-FN (fibronectin [FN]-grafted polystyrene [PS]) dishes were higher than those on PS or TCPS (tissue clture polystyrene) dishes. The presentation of specific binding site number of CS1 is 500 times (440,000/880) higher than that of fibronectin at the same grafting weight density of CS1 and fibronectin on dishes, because of low molecular weight of CS1 (approximately 880 dalton from 110 [average molecular weight of amino acids] x 8 [No. of amino acids in CS1]) than fibronectin (440,000 dalton). The presentation of specific cell-binding domains of CS1 at high concentrations seems to be important for the signal transduction that promotes the *ex vivo* expansion of HSPCs.

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IN VIVO IMAGING OF DYNAMIC INTERACTIONS WITHIN THE HAEMATOPOIETIC STEM CELL NICHE

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The stem cell microenvironment (or niche) is a critical regulator of stem cell function. Haematopoietic stem cells (HSC) reside within the bone marrow and therefore have been traditionally inaccessible to direct observation. For this reason, despite numerous functional studies demonstrating a role of several bone marrow stroma cells in regulating HSPC function and number, still very little is known about the anatomical location of HSC during steady state and in response to several types of stimuli. Many functional studies over the years have pointed to osteoblasts, bone-lining cells responsible for matrix deposition during bone growth and remodelling, as regulators of the HSC pool. Moreover, several studies have indicated that multiple perivascular stroma cells actively influence HSC fate. It is an open question whether HSC firmly localise within a specific niche or whether they migrate between different niches, defined by distinct bone marrow microenvironments, where they receive different stimuli leading to their quiescence vs. proliferation, self-renewal vs. differentiation. Another open question is whether leukaemia development affects HSC-niche interactions and whether leukaemia stem cells (LSC) and HSC compete for niche space. Confocal and two-photon hybrid microscopy allows visualization of single transplanted HSC in the calvarium bone marrow of live mice. FACS-purified, ex-vivo labelled haematopoietic stem and progenitor cell (HSPC) populations can be visualised upon their initial arrival in the niche (homing) and following the first divisions (early engraftment). We showed that long-term repopulating (LT) HSPC selectively localize proximal to osteoblasts within a few hours from transplantation, whereas their progeny are more distal. By analysing homing and early engraftment patterns of mutant HSPC known to dominate or fail when in competition with normal HSC for bone marrow engraftment, and of wild type HSC transplanted

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into environments known to improve or hinder their function, we monitored dynamic interactions between HSC and the bone marrow microenvironment and collected further evidence that HSC and HSC progeny localization near osteoblasts correlates with positive transplantation outcome. Finally, we used a mouse model of acute myeloid leukaemia based on retroviral transduction of the MLL-AF9 oncogene and observed that leukaemic granulocyte-monocyte precursors (L-GMP), an established LSC population, share the same homing pattern of normal GMP rather than that of LT-HSPC. This indicated why MLL-AF9 LSC are insensitive to niche-derived signals that otherwise affect normal HSC function.

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DUAL FUNCTION OF CX32 IN HEMATOPOIESIS: MAINTENANCE OF CELL QUIESCENCE AND SUPPORT OF PROLIFERATION OF HEMATOPOIETIC STEM/PROGENITOR CELLS

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Membrane channel connexin (Cx) forms gap junctions that are implicated in the homeostatic regulation of various cellular functions, including growth control and differentiation, apoptosis, and the synchronization of electrotonic and metabolic functions. Our studies of the role of Cx32, a member of the Cx family, in steady-state hematopoiesis and its potential protective role against leukemogenesis were reported previously (Exp Biol Med 232:700-12, 2007; J Membr Biol 217:105-13, 2007). Namely, in wild-type mice, Cx32 expression was detected in primitive hematopoietic stem/progenitor cells by immunocytochemistry and reverse transcriptase-polymerase chain reaction analysis, although it was not detected in unfractionated bone marrow cells. Cx32-knockout (KO) mice showed decreased numbers of peripheral mononuclear cells and various progenitor cell compartments, an increased number of cells in primitive stem cell fractions, such as the lineage marker (-)/c-kit (+)/stem cell antigen-1(+) (LKS) fraction, and a clearly delayed regeneration of progenitor cells after chemical abrasion. Furthermore, the incidence of methyl nitrso-urea (MNU)-induced leukemia was markedly high in the Cx32-KO mice after a single administration of MNU. Subsequent cell-cycle analysis of the LKS fraction using a cell sorter with Hoechst 33342 showed a significant increase in the percentage of the S phase fraction in the Cx32-KO mice. In this study, we examined the cell cycle of hematopoietic stem/progenitor cells (GM, CFU-GM; S9, CFU-S9; and S13, CFU-S13) by evaluating cycling stem/progenitor cells with continuous incorporation of bromodeoxyuridine (BrdUrd) *in vivo* for up to 6 weeks followed by exposure to ultraviolet A to eliminate only BrdUrd-incorporating cells (Exp Biol Med 227:474-9, 2002). The Cx32-KO mice showed an increased rate of percent kill (percentage of BrdUrd-incorporating stem/progenitor cells) of S13 during the initial 1 hr (S phase fraction); a prolonged doubling time of S13 and S9 or a shortened doubling time of GM; and an increased rate of percent kill at plateau level (entire cycling fraction) of S13 and GM. These findings indicate the dual function of Cx32 in hematopoiesis, i.e., one is the restoration of stem/progenitor cell quiescence and maintenance of primitive stem cells to prevent exhaustion, and the other is the support of stem/progenitor cell proliferation. Without Cx32, the percentage of the entire cycling fraction including the S phase fraction of not only the LKS fraction but also S13 increased, whereas simultaneously, doubling time of both S13 and S9 decreased. In contrast, GM showed a rather shortened doubling time and also a higher percentage of the cycling fraction in the Cx32-KO mice than in the wild-type mice, which might be regulated much strongly by the positive feedback from the decreased numbers of peripheral mononuclear cells than the direct Cx32 function. Moreover, these findings may provide a good explanation for the cumulative incidence of MNU-induced leukemogenesis in Cx32-KO mice, which was higher but increased more slowly than that in the wild-type mice.

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EXPRESSION OF EBF2 IN IMMATURE OSTEOBLASTIC CELLS DEFINES A NICHE FOR HEMATOPOIETIC STEM CELLS

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Hematopoietic stem cells (HSC) need a specialised microenvironment to maintain hematopoiesis life-long. Several cell types like osteoblasts, osteomacs, adipocytes, CXCL 12 abundant reticular (CAR) cells and nestin-positive mesenchymal stem cells have been implicated in the support of HSC. Among those, the strongest evidence for a functional involvement exists for osteoblastic cells, but the exact nature of HSC supporting osteoblastic cells is not clear. Early B cell factor 2 (Ebf2) is a transcription factor expressed specifically by mesenchymal stem cells, immature osteoblastic cells (IEO) and adipocytes of the bone marrow. Deficiency for Ebf2 does not alter the number of these cells, but their ability to support HSC and immature hematopoietic progenitors. *In vitro* studies suggest that the HSC supporting function is mediated via its expression in osteoblastic cells, but the differentiation stage as well as the contribution of other cell lineages like MSC and adipocytes is unclear. To analyse the cellular contribution to HSC support exactly, we generated a conditional Ebf2 knockout mouse line. At this point, we were successful in generating such a line, as ubiquitous deletion of the Ebf2flox allele fully recapitulates the phenotype of Ebf2-mutant animals. Surprisingly, deletion of Ebf2 in adipocytes (Ap2-Cre) does not influence HSC frequency to a detectable level, whereas deletion in immature osteoblastic cells (Osterix-Cre) fully recapitulates the phenotype of the conventional deletion of Ebf2. Furthermore, deletion of Ebf2 using the Prx1-Cre line, which supposedly is active in mesenchymal stem cells, does not lead to any phenotypic alterations. Taken together, the data show that Ebf2 is required for HSC maintenance specifically via its expression in immature osteoblastic cells, thereby defining these cells as components of a niche for HSC. As Ebf2 is a transcription factor, this finding also opens up the possibility of systematically analysing the transcriptional profile and target genes of Ebf2 in primary niche cells.

Mesenchymal Stem Cell Differentiation

Poster Board Number: T-2141

MECHANICAL DERIVATION OF FUNCTIONAL MYOTUBE FROM ADIPOSE DERIVED STEM CELLS

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In recent years, ECM stiffness and resulting cell contractility have been identified as potent stem cell differentiation regulators. Suc-

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cessful stem cell-based therapies will require acclimating cells to the abnormally stiff ECM of muscular dystrophy while inducing and/or maintaining myogenesis, fusion, and dystrophin delivery. Here we directly compare ASC to BMSC stiffness responsiveness and show myotube formation derived from ASCs on matrices that mimic skeletal muscle. ASCs are shown here to not just simply reflect the qualitative stiffness sensitivity of bone-marrow-derived stem cells (BMSCs) but to exceed BMSC myogenic capacity (40-fold higher myogenic marker expression on myogenic stiffness), expressing the appropriate temporal sequence of muscle transcriptional regulators on muscle-mimicking extracellular matrix in a focal adhesion- and contractility-dependent manner. 2% of ASCs formed multi-nucleated myotubes with a continuous cytoskeleton (10-fold higher than chemical induction) that was not due to misdirected cell division; microtubule depolymerization severed myotubes, but after washout, ASCs re-fused at a rate similar to pre-treated values. BMSCs never underwent stiffness-mediated fusion. ASC-derived myotubes, when replated onto non-permissive stiff matrix, maintain their fused state. Fusion frequency was increased by a contractile agonist, lysophosphatidic acid and decreased by a myosin inhibitor, blebbistatin. ASCs generated higher tangential force than BMSCs and showed more non-muscle myosin IIb. Mechanical induction was mediated via focal adhesions; vinculin assembled earlier in ASCs. Inhibiting fibronectin-integrin binding using alpha 5 or V integrin siRNA blocked mechanosensing process as ASCs fail to 'feel' myogenic 10kPa gel and to show myogenic mRNA expression. Together these data imply enhanced mechanosensitivity for ASCs, making them a better therapeutic cell source for fibrotic muscle.

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CONTROLLING STEM CELL FATE WITH SPATIALLY VARYING EXTRACELLULAR MATRIX STIFFNESS

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Regenerative musculoskeletal applications have been plagued with setbacks owing in part to the fibrosis present in degenerative muscle disorders, which increases collagen density and transglutaminase activity that stiffens the extracellular matrix (ECM). Injection of undifferentiated stem cells into diseased muscle, i.e. cellular myoplasty, was thought to ameliorate the disease, but instead causes cell calcification due at least in part to the abnormally stiff environment. Here, we have developed a patterned hydrogel that can assess stem cell phenotype maintenance and engraftment potential in a fibrotic-like environment *in vitro*. This "Zebraxis" bioreactor contains alternating regions of firm (10 kPa; myogenic) and stiff (34 kPa; fibrotic or osteogenic) or soft (1 kPa; neurogenic) and firm that mimic fibrotic muscle or innervated, healthy skeletal muscle, respectively. In the non-fibrotic model (1 vs. 10 kPa), C2C12 myoblasts migrate onto 10kPa stripes after day 1 and formed myotube by day 7 in lower serum conditions. Adipose-derived stem cells (ASCs), which we have previously shown are myogenic on firm ECM, also migrated onto firm stripes and fused on the non-fibrotic model. Supporting cell types on soft regions, e.g. PC12 neurons, should help this process. With the fibrotic model (10 vs. 34 kPa), ASCs and C2C12 myoblasts segregated on 34 and 10 kPa make direct contact and will mimic cellular myoplasty in vitro. Using ASC-derived myotubes-rather than undifferentiated stem cells-this in vitro system highlights our ability to monitor engraftment potential improvements in vitro using novel fibrosis-mimicking tissue equivalents. Our efforts to develop tissue engineered musculoskeletal systems hinge on the understanding gained here about the stem cell-microenvironment interaction so that we may overcome the fibrosis problem.

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RAPMYCIN ATTENUATES OSTEOGENIC PROPERTIES OF DAXAMETHASONE ON HUMAN BONE MARROW DERIVED MESENCHYMAL STEM CELLS

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Introduction: Application of allograft Mesenchymal Stem Cells in combination with immunosuppressive drugs which have osteogenic properties, would be a very good option to regenerate bone defects in clinics. Rapamycin, and Dexamethasone in particular, are two commonly used immunosuppressive drugs in clinics which have been shown to possess osteogenic properties. The aim of this study was to assess osteogenic properties of Rapamycin in combination with dexamethasone on promotion of osteogenesis in human bone marrow- derived Mesenchymal Stem Cells. Metods: To do that, Bone marrow-derived mesenchymal stem cells were cultured in osteogenic medium supplemented with 10nM Dexamethasone without rapamycin(Control) and with 1, 10, and 100 nM rapamycin, then evaluated at 7, 14 and 21 days post induction. Osteoblastic differentiation was characterized quantitatively by Alkaline Phosphatase Activity and Alizarin Red Staining Assay. Results: Our pilot study results showed that rapamycin alone showed no remarkable osteogenic differentiation effect on human mesenchymal stem cells, therefore we evaluated the synergistic effect of rapamycin and dexamethasone in promotion of osteogenesis. Our results showed that at day 7, Alkaline Phosphatase enzyme activity significantly increased in groups treated with Dexamethason and 1nM rapamycin rather than dexamethasone alone(Control). Two weeks post induction, the enzyme activity increased significantly in all groups in comparison with the same groups at day 7; however, the enzyme activity was remarkably decreased in groups treated with Rapamycin in comparison with Dexamethasone alone, and there was not a remarkable difference in enzyme activity in groups treated with 1, and 10 nM Rapamycin. At day 21, no significant difference was observed between groups which received both rapamycin(1 & 10nM) and Dexamethasone in one hand, and the control group which received dexamethasone alone on the other hand; however in presence of 100nM of rapamycin the enzyme activity was the least among he others. Evaluation of mineralization by alizarin red- based osteogenesis assay showed no difference between either control group(Dexamethasone alone) or other treated groups (Rapamycin & Dexamethasone) at day 7. However, one week later, matrix mineralization increased significantly in control group(Dexamethasone alone) in comparison with groups which received 10 & 100 nM of Rapamycin . At third week of induction, control group(Dexamethasone alone) showed a remarkable increase in matrix mineralization in comparison with other groups. Conclusion:Our results showed that Rapamycin would not be a good stimulator for promotion of osteoblastic differentiation of human bone- marrow- derived mesenchymal stem cells in combination with Dexamethasone. Not only have any synergistic effect in induction of osteogenesis with Dexamethasone, rapamycin attenuates the inductive effect of Dexamethasone in human bone marrow- derived mesenchymal stem cells.

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THE EFFICACY OF PURMORPHAMINE IN PROMOTION OF OSTEOGENESIS IN HUMAN BONE MARROW- DERIVED MESENCHYMAL STEM CELLS: APPROPRIATE DOSE AND RESPONSE TIME.

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Background: The Osteogenic differentiation capacity of human mesenchymal stem cells for bone formation and regeneration, leads to establish different strategies in order to provide reproducible systems for induction and enhancement of osteogenesis in these cells. Besides growth factors, some small molecules, such as purmorphamine serve as chemical tools in promotion of osteogenesis ; however, there has been no exact evaluation on the appropriate dose and the response time of this molecule for promotion of osteogenesis. The aim of this ongoing study is to define the most appropriate dose and the response time of Purmorphamine , to evaluate its efficacy in the promotion of osteogenesis in human Bone Marrow Mesenchymal Stem Cells in vitro and *in vivo*. Methods: To do that, Bone marrow derived mesenchymal stem cells were cultured in high glucose Doulbeco's modified eagle medium supplemented with 10 nM Dexamethasone without purmorphamine (control) and with 1, 3, 5, and 10 µM of this small molecule and evaluated at 7, 14 and 21 days post induction. Results: Although ALP activity was low 7 days post induction, it increased significantly ($p< 0.05$) in cells treated with 3 µM of purmorphamine in comparison with control group. Interestingly, one week later, this enzyme activity remarkably increased in all treated groups, especially in groups which received 3 and 5 µM doses of this small molecule. The enzyme activity decreased again one week later at day 21, and no significant difference was observed between purmorphamine- treated groups and the control one. Interestingly, our Real-time PCR results showed that 5microM of purmorphamine significantly($p< 0.05$) increased the expression level of RUNX-2 , an early transcription factor of osteogenesis, Osteocalcin and Osteopontin, two important matrix protein transcripts at day 14 post induction; however, the groups which received 10 µM of purmorphamine showed significant over expression of osteocalcin and osteopontin, not earlier than the end of the third week. We also analyzed mineralization capacity of the cells with and without (control) purmorphamine. Our results showed that although a significant increase in matrix mineralization was observed in all treated and non- treated groups from day 7 up to 21 days post induction; this augmentation was not significant in comparison with control group at each day. Conclusion: As whole, this part of our study indicates that (1) events related to osteoblast differentiation, including increased ALP activity and bone specific gene expression are enhanced in hBMMSCs at 2nd week post induction by 5 µM purmorphamine. (2) Due to high activity of ALP and over expression of bone specific genes such as RUNX-2 in the presence of 5microM of purmorphamine at day 14 and subsequent decrease in ALP activity and RUNX-2 expression at day 21; in order to *in vivo* study, we recommend to treat the cells with 5 µM purmorphamine at least for 14 days in transplantation.

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TRANSFORMING GROWTH FACTOR BETA 1 INDUCES THE DIFFERENTIATION OF RAM MARROW DERIVED MESENCHYMAL STEM CELLS INTO MALE GERM LIKE CELLS

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Many researchers have defined differentiation capacity of mesenchymal stem cells (MSCs). These cell types can be isolated from different sources such as bone marrow, fat tissue, neural system, lungs and muscles and can differentiate into very different cell types of all three germ layers. There are many reports about differentiation capacity of MSCs to mesoderm, ectoderm and endoderm cells and these findings have stimulated researchers to study the possibility of their application for treatment of some human diseases like diabetes, blindness, cancers, bone and cartilage problems and etc. In recent years one of the most attractive topics about MSCs was their application for infertility treatment. Some papers have shown a group of MSCs that have germ cells characteristics and some papers have shown MSCs capacity for differentiation into germ cells. The main design of these studies was based on treatment of MSCs by some materials (small molecules, growth factors, hormones and [[Unsupported Character - [[Unsupported Character - ⋯]]]]) *in vitro*. Although there are some good reports about the differentiation of MSCs to germ cells *in vitro* but this confusing issue needs more detailed and accurate studies. In this study we have shown, MSCs can be induced to differentiate into the male germ-like cells by TGFβ1. Materials and methods: Bone marrow aspiration was done from tibia of a newly mature ram under deep sedation condition that was induced by Ketamine and Xylazin injection. After use of density gradient (Lymphodex) and centrifuging (1200 rpm at 4°C for 20 min) mononuclear layer include MSCs was cultured in complete culture medium. Passage 3 MSCs used for treatment with TGFβ1. A 21 day treatment was done and after that the cells were examined by RT-PCR and real-time RT- PCR for germ cell-specific genes expression. The genes examined were OCT4, Vasa, piwil2, Dazl and β¬1integrin and immunocytochemistry was done for evaluation of PGP9.5 - the spermatogonia specific gene- expression. Quantitative alkaline phosphatase (ALP) activity assay was done too. In addition, treated cells were evaluated for cartilage specific genes, Col [[Unsupported Character - [[Unsupported Character - [[Unsupported Character - Ӏ]]]]]][[Unsupported Character - [[Unsupported Character - [[Unsupported Character - Ӏ]]]]]] and Aggrecan to ensure that no chondrogenic differentiation has happned. Results: Real-time PCR analysis showed upregulation of Vasa and β1 integrin and downregulation of OCT4 and Piwil2. Dazl expression wasn't seen in MSCs after 21 days treatment by RT-PCR. ALP activity increased by TGFβ1 treatment and high expression of PGP9.5 was observed in cells after treatment in compare with control group. Discussion: Although Dazl expression was seen in the sheep testis cells, it wasn't seen in 21 days treated MSCs. Totally, due to the increase of Vasa and β¬1integrin expression and down regulation of OCT4, gene expression pattern of sheep testis germ cells, germ cell gene expression pattern during germ cells development, increased ALP activity in MSCs after treatment that is high in germ cells too, no expression of cartilage specific genes and PGP9.5 high expression, we can conclude that TGFβ1 induced germ cell differentiation in sheep marrow-derived MSCs.

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EVALUATION OF DIFFERENT CONCENTRATIONS OF RETINOIC ACID IMPACTION ON GERM CELL SPECIFIC GENES EXPRESSION IN RAM MARROW DERIVED MESENCHYMAL STEM CELLS

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Introduction: Mesenchymal stem cells (MSCs) can be isolated from several sources and have high capacity for differentiation to different cells types of all three germ layers. In recent years some studies have discussed about the application of MSCs for germ cells (GCs) production *in vitro* and infertility treatment especially in males. Some researchers have suggested that some materials like some hormones, growth factors, retinoic acid and[[Unsupported Character - ⋯]] can induce GCs differentiation from MSCs *in vitro*. In this study we evaluated and compared effects of three different concentrations of retinoic acid (RA) treatment on male GC-specific genes expression in bone marrow-derived MSCs in sheep. Our main goal was to obtain germ cells from MSCs by RA treatment. Material and methods: MS like cells was isolated from an aspiration of bone marrow from tibia of a newly mature male sheep under deep sedation condition that was induced by Ketamine and Xylazin injection. The cells mesenchymal stemness characteristics confirmed by test of their osteogenic, adipogenic and chondrogenic capacity of differentiation. Passage 3 MSCs were cultured in complete media culture containing three different concentrations of RA for 21 days. RA concentrations were 1 µM (RA1), 5 µM (RA2) and 10 µM (RA3). At the end of the experiment time the cells were analyzed with RT-PCR and real-time RT-PCR for expression of male GS-specific genes and were immunostained for PGP9.5 - spermatogonia specific gene - expression. The genes examined for RT-PCR and real-time RT-PCR were OCT4, Vasa, piwil2, Dazl and β¬1integrin. Quantitative alkaline phosphatase (ALP) activity assay was done too. Results: Real-time PCR analysis showed upregulation of Vasa and β¬1integrin and downregulation of OCT4 and Piwil2 in all three groups. The greatest effect on genes expression was seen in RA3 group. Dazl expression was negative in 21 days treated MSCs. ALP activity increased by RA treatment but the amount of ALP in RA2 was greater than RA1 and RA3 groups and RA3 was the lowest. Expression of PGP9.5 increased in 21 days treated cells in compare with control group. PGP9.5 expression pattern was dose dependent and increased with increase of RA concentration. Discussion: Although Dazl expression was seen in the sheep testis cells, it wasn't seen in 21 days RA treated MSCs. Totally, given the increase of Vasa and β¬1integrin expression and down regulation of OCT4, gene expression pattern of sheep testis germ cells, germ cell gene expression pattern during germ cells development, overall increase of ALP activity in MSCs after treatment that is high in germ cells too and high expression of PGP9.5, maybe it could be concluded that RA can induces germ cell differentiation in sheep marrow-derived MSCs and the maximum effect was seen in 10 µM RA concentration .

Poster Board Number: T-2147

THE EFFECT OF THREE-DIMENSIONAL PILLAR SURFACES ON CELL ADHESION AND OSTEOGENIC DIFFERENTIATION

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Introduction The hypothesis was that compared to conventional two-dimensional (2D) cultures, substrates containing 3D nano- and micron-size pillars would allow cultured cells to grow at two or more different levels and help them attach to and extend e.g. from top-to-top or top-to-bottom, activating their cytoskeleton to promote osteogenesis. Materials and methods Fibroblasts, osteoblast-like SaOS-2 cells and human mesenchymal stem cells (MSC) were studied. Plain substrates were compared to 200 nm, 5 µm and 20 µm high pillars fabricated from Si or Ormocomp®. Some of the Si pillars were coated with diamond-like carbon (DLC) or TiO2. Adhesion of cells was evaluated with scanning electron microscopy (SEM) and staining of actin cytoskeleton. Activation of cytoskeletal tension was studied by staining extracellular signal-regulated kinases (ERK) and Rho-associated protein kinase (ROCK). Osteogenic markers, alkaline phosphatase (ALP), osteopontin (OP) and mineralization (Alizarin Red, OsteoimageTM) were stained to visualize the effect of pillars on osteogenesis. Results and discussion SEM and immunofluorescence staining of actin cytoskeleton of hMSCs and fibroblasts showed adhesion to high pillar edges at 7.5 hours and stretching between adhesion contacts at different levels well over 100 µm distances at 5 days. SaOS-2 cells, which are about half the size of hMSCs or fibroblasts, adhered flatly and individually on horizontal and vertical substrate surfaces without showing respect for contact inhibition. ERK and ROCK immunostaining verified the activation of the cytoskeleton in the stretched cells at 14 and 21 days. In contrast to expectations, the success of induced osteogenesis was thoroughly dominated by the cytocompatibility of the substrate, with early ALP, intermediate OP and late mineralisation markers, together with bone nodule formation, being seen in plain and low profile TiO2 pillars, but being poor in the 20 µm landscape. Only cell-high 5 µm pillars slightly enhanced induced osteogenesis. In 2D cultures, MSCs grew to confluence, with extensive cell-to-cell contact on their periphery allowing intercellular communication, whereas in 3D pillar surroundings cells were stretched between their two or more substrate contact points, with most of their cellular surface bathing in cell culture fluid. This lack of intercellular cell-cell contacts seems to suppress the osteogenesis promoting effects of cytoskeletal organization and tension, which osteogenesis promoting effects have otherwise been shown in many other experimental settings.

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Poster Board Number: T-2148

PULSED ELECTROMAGNETIC FIELD PROMOTES PROLIFERATION OF HUMAN MESENCHYMAL STEM CELLS AND DEHYDROEPIANDOSTERONE ENHANCES OSTEOGENESIS

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Introduction: The stem cell fate is determined by soluble factors, physical forces, cell-cell and cell-extracellular matrix contacts. We studied the effect of pulsed electromagnetic field (PEMF) and dehydroepiandosterone (DHEA) on the proliferation of human mesenchymal stem cells (hMSC) and osteoblastic SaOS-2 cells and on the osteogenic differentiation of hMSCs. The ability of stem cells to differentiate into bone cells is critical for fracture healing and integration of implants into bone. PEMF has been clinically used since food and drug administration approval in 1979 to promote bone fracture healing and others bone disorders for at least three decades its applications include osteoporosis, bone fractures, pseudoarthroses and osteotomies. The cellular mechanism by which the PEMF exerts its effects is still mainly unknown. DHEA is a prohormone produced in the reticular zone of the adrenal cortex, but only in man and other primates. It is in peripheral cells and tissues metabolized by steroidogenic, intracrine enzymes to various active sex steroids, e.g. 17ß-estradiol (E2) or dihydrotestosterone (DHT). Such functionally active sex steroids play key roles in bone metabolism and their failure is a well-recognized cause of post-menopausal osteoporosis in women and osteoporosis in hypogonadism in men. Materials and methods Human osteogenic sarcoma SaOS-2 (ECACC 890500205) cells and bone marrow derived hMSCs (PoieticsTM, Lonza, Basel, Switzerland) were cultured. PEMF were produced by OSSATEC® Bone growth stimulation device (Uden, The Netherlands) using continuous stimulation. To test the effect of DHEA, the cells were cultured in 1, 10 µM or 100 µM DHEA (Sigma Aldrich, St. Louis, MO, USA), with and without 100 nM dutasteride (Avodart®, GlaxoSmithKline), which inhibits type I and II 5-alpha-reductase. Proliferation was studied using the MTT-method at days 4-21 and differentiation at days 1-28 with quantitative real time polymerase chain reaction and staining of osteogenic markers. Results and discussion PEMF promoted the proliferation rate of both cell types and DHEA decreased it in a concentration dependent manner. PEMF stimulation increased hMSC proliferation by 16% at day 14 and SaOS-2 proliferation by 30% at day 11 compared to non-stimulated cells. DHEA treatment decreased the proliferation rate of hMSCs by 58% at day 21 and proliferation of SaOS-2 cells by 35% at day 14 compared to non-stimulated cells ($p < 0.001$ for all comparisons). DHEA clearly promoted the osteogenesis of hMSCs, which were induced to osteogenesis with beta-glyserophosphate, ascorbate and dexamethazone. Alkaline phosphatase, SMAD1, RUNX2, osteocalcin (OC) and osteopontin (OP) RNA levels were increased and Alizarin red S and hydroxyapatite specific OsteoImageTM stainings showed mineralization. In addition, DHEA increased OC and OP mRNA levels of non-induced hMSCs. At this low seeding density, 3000 cells/cm2, PEMF did not have major effects on osteogenesis, which is logical as proliferation and differentiation are generally considered opposite and mutually exclusive states in cell biology, with proliferation being followed by differentiation. Conclusions PEMF stimulated the proliferation of hMSCs and SaOS-2 cells and DHEA stimulated the osteogenic differentiation of hMSCs.

Poster Board Number: T-2149

EXPANSION AND DIFFERENTIATION OF HUMAN MESENCHYMAL STROMAL CELLS

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Human mesenchymal stromal cells (hMSC) are candidates for clinical use because they are readily expanded in culture, have immuno-modulatory potential and can differentiate into the osteogenic, chondrogenic and adipogenic lineages. Their therapeutic potential is currently studied as part of clinical trials to treat diseases such as graft-versus-host disease and osteoarthritis, as well as in the regeneration of cardiac muscle following myocardial infarcts. Whether the requirements are for clinical or research use, obtaining a substantial number of cells can constitute a bottleneck for the investigator. hMSC display some plasticity in their culture conditions, but several investigators report a higher growth index and increased differentiation potential at lower seeding densities. We present here a protocol enabling the clinician or researcher to rapidly expand a population of hMSCs on Thermo Scientific Nunclon Delta cell culture treated surface utilizing the potential of Thermo Scientific HyClone AdvanceSTEM Mesenchymal Stem Cell Basal Medium, developed specifically for the optimal expansion and maintenance of undifferentiated hMSCs. A definite test of multi-potency is a functional test. In consequence, we subjected the expanded hMSC to differentiation. The cells were differentiated into osteoblasts or adipocytes in Nunc 48 well multidishes.

Poster Board Number: T-2150

EFFECTS OF VALPROIC ACID ON THE EXPRESSION OF NEUROTROPHIC FACTORS IN HUMAN BONE MARROW MESECHYMAL STEM CELLS

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Human bone marrow-mesenchymal stromal cells (hBM-MSCs) can differentiate into diverse cell types and secrete a variety of trophic factors, which provides an excellent cell therapy tool for disease models. However, limited efficacies have been reported in autologous or allogeneic stem cell transplantation. For the improvement of efficacy of stem cell therapy, the optimized conditions of stem cells need to be defined. In this study, we have evaluated the effects of valproic acid, known as HDAC inhibitor, in human BM-MSCs and identified the increment of trophic factors including *ANG*, *BDNF*, *ECFG*1, *GDNF*, *HGF*, *IGF*, *PIGF*, *TGF*-1β, and β-*Pix* in 200 µg/ml VPA treated MSCs for 12 hours. The MSCs were not changed their features in this conditions. MSCs also have presented the increased cell protective effect against oxidative injuries tested by MTT assay and improved migratory ability examined by Boyden chamber in this VPA treated condition. It suggests that the natural deficit MSCs from unhealthy donor may be improved their capacities by treatment of VPA with optimal dose and time, which may help better efficacies in stem cell therapy.

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Poster Board Number: T-2151

EFFECT OF T3 ON CHONDROGENESIS *IN VITRO* OF HUMAN MESENCHYMAL STEM CELLS FROM UMBILICAL CORD STROMAL SOURCE

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There are evidences about the thyroid hormone role into chondrogenesis but its molecular implication is not clear. The current study treats to answer this question working about a new *in vitro* model of chondrogenesis using mesenchymal stem cells (MSCs) from human umbilical cord stroma tissues The umbilical cord tissues were obtained from caesareans from healthy women in the Maternity Facility at Complejo Hospitalario Universitario de A Coruña under the supervision of the hospital ethic committee. MSCs were isolated from umbilical cord stroma tissue using an enzymatic digestion and cultured the cells adhered to the plastic plate. Chondrogenic process was performed using our model which consisted in growing the cells during two days in medium with FCS 10% in DMEM. After 2 days the medium was replaced by a medium with KO serum and TGF-ß3 which induces the chondrogenesis through spheroid formation. Different amounts of human T3 (1, 10 or 100 ng/mL) were adding to the medium to test their role into chondrogenesis process, also different experiments with T3 alone or together with M151, a specific steroid covalent receptor type 2 (SCR2) inhibitor, in the chondrogenic medium, were performance to check which was the T3 receptor which had an active role in the chondrogenesis. Spheroids made by this method were collected after 7, 14 and 28 days in culture and storage at -20ºC for their posterior analysis. Immunohistochemistry analysis of spheroids against COL2, COL1 and aggrecane stain was performed to check chondrogenic differentiation stage and quantitative RT-PCR to check expression of COL2, COL1 and SOX9 genes also was made. Furthermore, proteins involved into Wnt (β-catenin and GSK3-β) and Notch (Notch2 and Jagged) pathways were analyzed by western. Immunohistochemistry analysis of the tissues from the spheroids demonstrated that 100 ng/mL of T3 improved significantly ($P<0.05$) the COL2 and aggrecane staining after 14 days into chondrogenic medium versus chondrogenic medium without T3. This improving was totally withdrawal when the SCR2 inhibitor, M151, was added to the medium at 2.5 ng/mL dose. Quantitative RT-PCR analysis confirm the immunohistochemistry results, improving significantly ($P<0.05$) the COL2 and aggrecane gene expression after 14 days into chondrogenic medium when 100 ng/mL of T3 was present and revealed that expression of COL1 was significantly increased ($P<0.05$) when M151 (2.5 ng/mL) was added to this medium. Western analysis indicated that β-catenin was increased in the spheroids when T3 (100 ng/mL) was in the medium after 14 days (2 fold) with respect to medium alone and was decreased until basal levels found in spheroids growth into medium without T3, when M151 (2.5 ng/mL) was present in the medium. Notch2 was increased (2 fold) during the chondrogenesis when T3 was present in the medium with respect to medium alone and the opposite effect was found when M151 was present in the medium. T3 is improving the chondrogenic differentiation of mesenchymal stem cells from umbilical cord tissue in our *in vitro* model. That effect is mediated through SCR2 as well as T3 is associated the expression of β-catenin and Notch2 in our chondrogenic model.

Poster Board Number: T-2152

MACROMOLECULAR CROWDING AND HUMAN MESENCHYMAL STEM CELL DIFFERENTIATION

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The *ex vivo* expansion of stem cells is an important step towards realizing the great potential of stem cell-based therapy in modern medicine. Stem cells are promising therapeutic agents because they could serve as a source of replacement tissues and organs. Cell-based therapies involve removing cells from a physiological environment and propagating them in an artificial cell culture system. A challenge in stem cell research today is ensuring that therapeutically adequate numbers of cells are produced for subsequent transfer to a patient. The non-physiological conditions to which cells are exposed during their lengthy *ex vivo* cultivation compromise their phenotype. Cells function less efficiently, resulting in sub-optimal proliferation and differentiation. The *ex vivo* differentiation of hMSCs into the adipogenic lineage is enhanced dramatically when a Ficoll mixture is used as a macromolecular crowder in the differentiation protocol. Macromolecular crowding is known to affect molecular diffusion and biochemical reaction rates. Fluorescence correlation spectroscopy (FCS) is a powerful single-molecule-sensitive technique that has been used extensively to study dynamic molecular processes in living cells and organisms. We have used FCS to probe diffusive events in different experimental systems. Our goal is to understand how Ficoll interacts with hMSCs and improves their proliferation and differentiation. We have found that molecular diffusion in the cell surface membrane and in supported lipid bilayers (SLBs) is not changed significantly by the crowder, suggesting that Ficoll does not directly alter membrane properties and cell surface signalling. We have measured the diffusion of fluorophore-labelled Ficoll 70 and Ficoll 400 in the cytoplasm of hMSCs as well as in solutions of physiologically relevant concentrations of unlabelled Ficoll 70 and Ficoll 400. By using an appropriate data fitting model, we are able to describe the mode of diffusion occurring in the cytoplasm and solutions. Ficoll 70 and Ficoll 400 undergo anomalous subdiffusion (ASD) in the cytoplasm of hMSCs. In the solutions, however, the degree of anomalous subdiffusion is lower than it is in cytoplasm. However, we have data that conclusively show that the same concentrations of Ficoll 70 and Ficoll 400 increase actin polymerization reaction rates in vitro. We have also determined that Ficoll increases the rate of in vitro collagen aggregation. As collagen is a principal component of the extracellular matrix (ECM), we hypothesize that the excluded volume effect caused by Ficoll in the stem cell culture media directly affects the rate of deposition of ECM. Based upon our data, we conclude that Ficoll crowders have a greater effect on biochemical reaction kinetics than on molecular diffusion. Ficoll is thus an effective modulator of the *ex vivo* behaviour of hMSCs.

Poster Board Number: T-2153

ISOLATION AND CHARACTERIZATION OF MESENCHYMAL-LIKE STEM CELLS FROM HUMAN SALIVARY GLAND

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Salivary glands produce and secret saliva. Stem cells of salivary gland are known to regenerate and ameliorate on their function after damage. Here we report that salivary gland stem cells were isolated from human salivary glands. In order to effectively isolate and

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amplify stem cells in large amounts from submandibular glands, we developed a culture system (lasting four to five weeks) without any selection. After five passages, we found adherent cells which have expressed MSC surface antigen markers such as CD44, CD49f, CD90 and CD105, not including CD34 and CD45 hematopoietic stem cell marker. As we expected, these cells were differentiated into adipogenic, osteogenic, and chondrogenic cells with tissue-specific induction medium. Finally, the mesenchymal-like stem cells were isolated from submandibular gland and have great potential to can be used to regeneration of other organ.

Poster Board Number: T-2154

ANALYSIS OF THE PROSPECTIVE CELL SURFACE MARKERS FOR HUMAN MESENCHYMAL STEM CELLS THAT READILY DIFFERENTIATE INTO CARDIOMYOCYTES.

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Myocardial infarction caused by chronic atherosclerosis of the coronary artery could induce heart failure with high mortality. Because myocardium has very limited ability of regeneration, heart transplantation is currently considered to be the most effective treatment for severe heart failure. In the case of patients with some remaining heart functions, ventricular assist devices can be used to complement their lost functions. For severe patients who need to replace their heart, artificial hearts are used in order to bridge the time to heart transplantation for a limited period of time. However, at present these devices cannot be used forever, and long-time antithrombogenicity, biocompatibility, reliability and durability are needed to be improved. On top of that, limited number of donor for heart transplantation causes serious issues of this methodology. Recently, stem cell therapy is expected to be the alternative regenerative medicine for heart failure. In addition to pluripotent embryonic stem (ES) cells and induced pluripotent stem (iPS) cells, Mesenchymal stem cells (MSCs) are one of the most promising stem cell sources for regenerative medicine. MSCs have been studied extensively and shown to differentiate into various cells including bone, cartridge, fat, neuron, skeletal muscle, and cardiac muscle lineages *in vitro*. MSCs can readily differentiate into osteoblasts, chondrocytes and adipocytes, but they do not differentiate into the other type of tissue cells, such as cardiomyocytes, at high efficiency. Recent reports suggested that delivery of adult MSCs into ischemic tissue augments the recovery of cardiac function after ischemia *in vivo*. The effects induced by MSCs are generally temporary and limited.In this study, we investigated prospective cell surface markers that enable to enrich cardiomyogenic progenitors from MSCs derived from human bone marrow or adipose tissues. We analyzed the cell-surface expressions of trans-membrane protein markers that are essential during heart development using various human MSCs and various human MSC cell lines were co-cultured on mouse cardiomyocytes. These differentiated cells expressed terminally differentiated cardiomyocyte-specific markers, such as α-actinin, cardiac troponin T, and connexin-43. We found that cell-surface expression of N-cadherin shows the best correlation to the transdifferentiation potential into cardiomyocytes. In contrast, MSC cell lines with low expression of N-cadherin showed apparently less differentiation abilities toward cardiomyocytes. Similar correlation was also observed with primary human MSCs derived from bone marrow and adipose tissue. We further demonstrated that the cell population with higher cardiomyogenic potential can be isolated from heterogeneous primary MSCs using N-cadherin antibody immunoaffinity beads. N-cadherin-high MSC population separated by anti-N-cadherin antibody beads revealed significantly higher expression of Nkx2.5 and GATA4 mRNAs and higher differentiation abilities into cardiomyocytes than those of N-cadherin-low expression. Our results suggested that N-cadherin could be a prospective cell surface marker for MSCs that have higher cardiomyogenic potential.

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CHARACTERIZATION OF OSTEOPROGENITORS FUNCTIONALLY ISOLATED FROM HUMAN MESENCHYMAL STEM CELLS BY A RUNX2-RESPONSIVE REPORTER ADENOVIRUS

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Objective: Mesenchymal stem cells (MSC) are a heterogeneous cell population characterized by their self-renewal capability and their multidifferentiation potential. Current isolation methods of MSCs are still rudimentary due to the lack of a unique marker. Here, we report a novel method for the isolation of osteoprogenitors from human MSCs, along with the characterization of the resulting cell populations. A subpopulation of MSCs was functionally identified and isolated by coupling the expression of the key osteogenic transcription factor Runx2 to the expression of enhanced green fluorescent protein (EGFP) via a Runx2 reporter adenovirus. On that basis, fluorescing cells can be selected by means of fluorescence activated cell sorting (FACS). Material and methods: MSCs were obtained from bone marrow aspirates by Ficoll separation and cell attachment to plastic. MSCs were expanded in the presence of bFGF. Cells were infected with the Runx2 reporter adenovirus using lanthofection at 100 MOI. Cells were then subjected to osteogenic induction for 3 days and sorted by means of FACS. The resulting cell populations, namely Runx2 GFP+, Runx2 GFP-, and the unsorted cells, were separately expanded in the presence of bFGF, and thereafter subjected to comparative investigation of their in vitro osteogenic differentiation potential. Characterization of the cell populations was substantiated with the assessment of their proliferative capacity. Osteogenic differentiation was evaluated by alkaline phosphatase (ALP) activity at d7, 14, and 21, as well as 45Ca incorporation at d21. To perform the functional analysis of the Runx2 binding sites, a Runx2-driven luciferase reporter plasmid as well as Runx2 expressing and non-expressing effector plasmids were prepared. Results: Colony forming unit (CFU) analysis at d14 revealed that Runx2 GFP+ cells proliferate at a slower rate than the other two cell populations. This suggests that Runx2 GFP+ cells show a more committed/differentiated phenotype than the other two cell populations. ALP activity of Runx2 GFP+ cells was shifted towards earlier timepoints of osteogenic differentiation, showing highest ALP activity at d7 as opposed to d14 for the other two cell populations. 45Ca incorporation to assess matrix mineralization was massively higher for osteogenically differentiated Runx2 GFP+ cells than for the other cell populations treated with the same medium. Results of both assays are in accordance with each other, indicating that Runx2 GFP+ cells are more osteogenic than Runx2 GFP- and unsorted cells. Further, we investigated the functionality and specificity of the reporter by means of luciferase assay at 48 hours post-transfection. Upon cotransfecting HeLa cells (which do

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not express Runx2) with a Runx2 expressing effector plasmid and a luciferase reporter plasmid driven by the same Runx2 binding sites used in the Runx2 reporter adenovirus, a clear transactivation can be seen compared to the Runx2 non-expressing cotransfection control. These findings indicate that the Runx2 binding sites are responsive to Runx2 protein. Conclusion: We have utilized a Runx2-responsive reporter adenovirus to sub-divide osteogenically induced human MSCs. Reporter-positive subpopulation displays characteristics appropriate for osteoprogenitors: (1) a slower proliferation rate, and (2) a more osteoblast-like phenotype upon in vitro osteogenic differentiation, as compared with reporter-negative as well as original cell population.

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MODULATION OF HUMAN MESENCHYMAL STEM CELL BEHAVIORS BY NANOSCALE GROOVE PATTERN TOPOGRAPHY

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Cell-substrate interactions are crucial for controlling diverse cellular behaviors. In particular, surface topographical effects of culture substrate are known to modulate adhesion, alignment, proliferation, and differentiation of stem cells. In this study, we investigated the effects of nanoscale topographies on the behaviors of human mesenchymal stem cells (hMSCs). Human adipose tissue-derived MSCs were cultured on polyurethane acrylate (PUA) nanopatterned surfaces (150-300 nm in width, 300-600 nm in pitch, and 200 nm in depth) grafted with glycidyl methacrylate (GMA) by initiated chemical vapor deposition (iCVD) technique. The surfaces of PUA substrates were modified with GMA group for potential grafting of bioactive molecules with functional groups that have the binding affinity to GMA. Live/Dead staining assay revealed that there was no significant difference in hMSC viability and proliferation between GMA-coated and uncoated PUA nanopattern groups, indicating that iCVD-mediated GMA coating did not cause significant cytotoxicity. The morphological changes of hMSCs cultured on the GMA-coated PUA groove nanopatterned surfaces were examined by F-actin cytoskeleton (phalloidin) staining and compared with those of hMSCs on the GMA-coated PUA flat surfaces. Interestingly, the culture on the groove nanopatterned surfaces significantly promoted elongation and alignment of hMSCs along with the axis, whereas hMSCs cultured on the flat surfaces did not exhibit such elongated and oriented morphology. Given that GMA contains epoxy group that can readily bind to amine functional group, diverse bioactive molecules including growth factors and adhesion peptides can be easily immobilized onto the GMA-PUA nanopatterned surfaces. Therefore, combinatorial effects of surface topography and bioactive molecules will be examined for enhancing proliferation and lineage-specific differentiation of hMSCs in future study. Acknowledgment: This study was supported by grants (2010-0025982, 2010-0022037, and 2011-0027538) funded by the National Research Foundation of Korea.

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GENERATION OF PRE-ADIPOCYTES FROM HUMAN ADIPOSE DERIVED STEM CELLS AND HUMAN PERIPHERAL BLOOD STEM CELLS

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Mesenchymal stem cells (MSCs) are found throughout the body and can be harvested from many sources including, blood, bone marrow, and adipose tissue. Their multipotency enables them to give rise to a variety of different linages, such as, adipocytes, osteoblasts and chondrocytes. MSCs have been terminally differentiated into the above mentioned lineages using an array of techniques and commercially available kits. Current protocols, suggest the use of a cocktail of differentiation inducers to commit MSCs into certain cell lineages, such as, 3-isobutyl-1-methyl-xanthine, dexamethasone, insulin, and indomethacin to guide MSCs to the adipose lineage. A recent study also shows that adipose lineage commitment can be induced in murine embryonal MSCs by treatment with bone morphogenic protein-4 (BMP-4) alone. Thus, we hypothesize that stem cells isolated from blood and adipose tissue treated with BMP-4 will also demonstrate commitment to the adipose lineage. Exposure of MSCs to BMP-4 directs them to the adipocyte lineage by differentiating the MSCs into pre-adipocytes (PAs). PAs are adipocyte precursor cells that are fibroblast-like in nature and maintain the capacity for self-renewal. Their ability to self renew plays a central role in maintaining adipocyte populations in the body. The presence of intracellular lipid droplets, found in PAs and adipocytes, is a strong indicator a cell is from the adipose lineage. Oil Red O, a lysochrome diazo dye, is used to visualize the formation of lipid droplets within cells. Oil Red O staining of BMP-4 treated cells obtained from two sources, blood and adipose tissue, confirmed the presence of lipid droplets suggesting that BMP-4 alone can force commitment to the adipose lineage. However, both PAs and adipocytes contain lipid droplets, therefore to further identify whether the BMP-4 treated cells were PAs and not adipocytes, they were tested for markers specific to PAs. Antibody staining showed positive for the expression of PA markers, and were negative for adipocyte markers in the BMP-4 treated cells. In conjunction, these two staining methods demonstrate that adipose commitment is possible in cells obtained from blood or adipose tissue through exposure to BMP-4 alone. The ability to create PAs derived from blood and adipose tissue will eliminate the need for using bone marrow, a very invasive method of collecting MSCs. Additionally, the self-renewal potential of PAs makes them a good alternative to lipoinjection therapies. Current therapies use lipoaspirates that are heterogeneous cell populations and contain low numbers of adipocyte precursor cells. A low population of PAs fails to establish an adipocyte regeneration pool, making the effects of lipoinjecton short term. Thus, increasing the population of PAs before injection may increase the longevity of current lipoinjection procedures ensuring a more effective treatment.

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MITOCHONDRIAL ALTERATIONS DURING ADIPOGENIC DIFFERENTIATION OF HUMAN ADULT MESENCHYMAL STEM CELL

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Mesenchymal stem cells (MSCs) received increased attention due to their invaluable potential to differentiate into all kind of cell lineages that can replenish the cell pool. However little effort has been made in studying the metabolic aspects of stem cells. This importantly involves mitochondrial alteration i.e. mitochondrial number, membrane potential, ROS production and cellular redox status.

We studied the mitochondrial alteration including their membrane potential (MMP), cellular load, production of superoxides and peroxides during adipogenic induction of human adult mesenchymal stem cells. Human adult mesenchymal stem cells were isolated from adult human bone marrow after consent. These MSCs were characterized for the presence of stem cell surface marker i.e. CD90, CD105 and CD 73 along with the absence of hematopoietic lineage makers CD34 and CD45. They were successfully differentiated them into hepatocytes, oseocytes and adipocyte and characterized using lineage specific stains (glycogen storage using PAS stain and ICG uptake, alizarin-red, Oil Red -O stain respectively). For targeted study, MSCs were induced with adiopogenic medium (dexamethasone, insulin, IBMX, indomethacin) and the mitochondrial alterations (peroxide and superoxide levels, mitochondrial load and mitochondrial membrane potential) was analysed from day of induction to 21 days. All the mitochondrial alterations were analyzed by flowcytometery. Cells were incubated with DCFH-DA for peroxide and DHE for superoxide for 45mints at 37°C. For mitochondrial load and MMP cells were incubated with NAO for 30 mints and JC-1 for 10 mints at 37°C respectively within 24 hrs of induction. Peroxide level decreased by 12% and mitochondrial load by 17%. Peroxide production gradually decreased after 2nd day of differentiation whereas mitochondrial load remained unchanged throughout the differentiation period of 21 days. The superoxide level and mitochondrial membrane potential increased by 7% and 9% respectively within 24hr of induction, after that there was continuous decrease in the superoxide level with the progression of differentiation while the mitochondrial membrane potential decreased upto day 4 and after it become stable. Conclusion: Adipogenic induction decreased the peroxide levels, which are complemented by decreased mitochondrial load, and increased superoxide levels. The significance of these changes needs further studies. DCFH-DA: 2′, 7′ - dichlor[[Unsupported Character - ﬂ]]uorescein diacetate; DHE: dihydroethidium; NAO: 10-nonyl acridine orange; JC-1: 5, 5- , 6, 6- -tetrachloro1,1- ,3,3- -tetramethylbenzimidazolylcarbocyani ne iodide; IBMX:1-methyl-3-(2-methylpropyl)-7H-purine-2,6-dione; PAS: Periodic acid-Schiff ; ICG: indocyanine green.

Poster Board Number: T-2159

ENHANCEMENT DIFFERENTIATION OF HUMAN INFRAPATELLAR FAT PAD STEM CELLS IN A HYALURONAN-ENRICHED MICROENVIRONMENT

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Microenvironment plays a critical role in guiding stem cell differentiation. We investigated the effect of a hyaluronan (HA)-enriched microenvironment on chondrogenesis of human infrapatellar fat pad stem cells (IFPSC). The IFPSC were obtained from patients undergoing arthroscopy. HA-coated culture wells were used as HA-enriched microenvironment. The mRNA expression of chondrogenic, adipogenic and osteogenic marker genes were quantified by real-time polymerase chain reaction. Sulfated glycosaminoglycan (sGAG) deposition was detected by Alcian blue assay. The IFPSC cultured in HA-coated wells (HA concentration from 25 to 100%) showed enhanced aggregation and increased sGAG deposition. The migration of IFPSC was promoted under chondrocyte condition medium. Our results suggest that HA-enriched microenvironment induces chondrogenesis in IFPSC, which may be beneficial in articular cartilage tissue engineering.

Poster Board Number: T-2160

UTILIZING COMPARATIVE GENOME-WIDE EPIGENETIC AND TRANSCRIPTIONAL ANALYSIS TO IDENTIFY AND CHARACTERIZE NOVEL REGULATORS OF INFLAMMATION IN HUMAN PRIMARY ADIPOSE TISSUE.

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Over the past several years it has been increasingly recognized that obesity is a strong contributing factor to a number of human diseases including cardiovascular disease, cancer, and most notably, type II diabetes (T2D). One of the underlying features of the obesity-related insulin resistance observed with T2D is chronic low-grade inflammation of adipose tissue. This inflammation is characterized by a marked increase in macrophage infiltration into adipose tissue with concomitant pro- inflammatory cytokine secretion. While several recent studies have highlighted the link between obesity and inflammation, the signals and mechanisms that trigger and maintain the inflammatory response are not well understood. In order to better understand the role obesity is playing in inflammation-mediated T2D, it is imperative to better understand the regulation and function of immune modulating factors in adipocytes. Unfortunately there are currently no robust in vitro model systems to study adipose inflammation. A well-established model of adipocyte function is based on the use of cultured adipose derived mesenchymal stem cells (ADMSCs). ADMSCs can be physically transitioned from a fibroblast-like state to an adipocyte-like state using hormones and mitogens. Although this tool has been very helpful for studying the development and function of fat cells in vitro, ADSMC-derived adipocytes are not exposed to the immune factors found *in vivo*, therefore studying ADMSC-derived adipocytes alone is not an ideal model for understanding mechanisms of adipose tissue inflammation. To bridge this gap between in vitro and *in vivo* adipocytes we have devised a genome-wide epigenetic and transcriptional screen to identify novel regulators of adipose inflammation. By comparing sites of histone H3 lysine 4 methylation (H3K4me1, active promoter/enhancer mark) and whole genome transcriptional profiles between primary human adipocytes from several obese donors to donor-matched ADMSC-derived adipocytes we have identified a novel transcription factor which may play a role in regulation of inflammation-mediated insulin resistance. Ectopic expression of this single transcription factor in ADSMC-derived adipocytes leads to upregulation of several transcriptional pathways characteristic of an immune response. In addition, these adipocytes exhibit several robust phenotypes characteristic of inflamed adipose tissue *in vivo*, including lipid droplet hypertrophy, pro-inflammatory cytokine release, down-regulation of lipolysis, and insulin resistance. In conclusion, by integrating H3K4me1 ChIP-Seq data with microarray

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expression analysis from primary fat cells versus their in vitro derived counterparts, we have identified a transcriptional mediator of inflammation in adipocytes. ADMSC-derived adipocytes expressing this transcription factor may prove to be a useful tool for studying obesity-induced inflammation in vitro.

Poster Board Number: T-2161

DEVELOPMENT OF PREVASCULARISED DENTAL PULP CONSTRUCTS BY SYNERGISTIC EFFECTS OF HUMAN DENTAL PULP STEM/PROGENITOR CELLS AND ENDOTHELIAL CELLS

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Introduction: Dental pulp stem cells (DPSCs) have received much attention as a promising population of stem cells in regenerative endodontics. Securing a good blood supply during regeneration is a challenging task due to the constricted apical canal opening which allows only a limited blood supply. The objectives of this study were to investigate the synergistic effects of dental pulp stem cells (DPSCs) and endothelial cells (ECs) on osteo/odontogenic differentiation and vasculogenesis; and to fabricate prevascularised 3-dimensional pulp constructs in-vitro. Methods: Different ratios of DPSCs and ECs were cultured in direct-contact in optimized-medium and were induced for osteo/odontogenic differentiation up to 3-weeks. Alkaline phosphatase (ALP) activity, expression levels of ALP, bone sialoprotein (BSP), dentin sialophosphoprotein (DSPP) genes and Alizarin Red staining for mineralization at different time points were analyzed. Tubular network formation on Matrigel and gene expression levels of CD117, VEGF, CD34, Flk-1 were used to analyse vasculogenesis. Three-dimensional microtissue-spheroids of DPSCs and ECs were fabricated in agarose 3D petri-dishes made using 12-series micro-molds (MicroTissues Inc.). Briefly, the cell suspensions were added to the each 3D petri dish and allowed 10 minutes for cells to settle into the features. After incubating at 37oC in 5% CO2 atmosphere for 3 days, the petri dishes were inverted and centrifuged at 500 rpm for 5 minutes to harvest microtissue spheroids. Microtissue-spheroids (1200) were transferred to a custom-designed, 3mm-diameter, agarose mold and cultivated for 4-days to self-assemble into macrotissue. The macrotissues were induced for odontogenic differentiation (21-days), examined for mineralization (Von-Kossa) and for vascularisation (Immunohistochemistry for CD31). All experiments were conducted in triplicate using DPSCs from three different donors and statistically analysed using one-way ANOVA. Results: The quantification of ALP revealed significantly greater activity in DPSC:EC co-cultures compared to DPSC-alone cultures ($p<0.05$). DPSC:EC, 1:1 and 1:5 co-cultures had a greater amount of calcification under Alizarin Red staining compared to other cultures ($p<0.01$). The higher expression levels of ALP, BSP and DSPP genes further confirmed the greater osteo/odontogenic differentiation in co-cultures compared to those of DPSC-alone cultures. Matrigel assay showed that the addition of DPSCs stabilized pre-existing vessel-like structures formed by ECs and increased their longevity. Compared to DPSC-alone cultures, the co-cultures expressed higher levels of vasculogenic markers. DPSC-EC macrotissues showed a significantly higher amount of extracellular matrix and mineralization compared to DPSC-alone macrotissues in 3-D. In contrast to DPSC-alone macrotissues, a dense-network of ECs was found throughout the DPSC:EC macrotissues under immunohistochemical analysis for the EC-specific marker CD31. Conclusion: DPSCs and ECs synergistically enhance vasculogenesis and osteo/odontogenic differentiation; and prevascularize pulp-like macrotissues in-vitro.

Poster Board Number: T-2162

HEPATIC DIFFERENTIATION OF HUMAN DENTAL PULP STEM CELLS: TRANSPLANTATION INTO RATS WITH LIVER INJURY AND CIRRHOSIS

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Objectives: The stromal mesenchymal tissues offer a large therapeutic potential in the regenerative medicine. In this study our goal is to differentiate stem cells cultures from human tooth pulp to hepatic-like cells with serum-free medium and to prove that after transplantation into F344 nude rats' spleens, human hepatic tissues, including blood vessels and bile ducts, were reproduced. Methods: CD117+ cells were isolated from deciduous tooth pulp stem cells (SHED) cultures by magnetic separation. The CD117+ cells were purified at every 4 passages to keep the undifferentiated stem cells phenotype. Cultures were characterized for expressing a panel of stem cell markers by immunofluorescence. Cells were grown in DMEM supplemented with 50ng/ml insulin-transferrin-selenium-x (ITS-x), 100µg/ml embryo-trophyc factors (ETF) and 20ng/ml hepatocyte growth factor (HGF) for hepatic commitment (5 days). For hepatic differentiation the medium was changed to IMDM supplemented with 50ng/ml ITS-x, 100µg/ml ETF, 10ng/ml oncostatin, 20ng/ml HGF and 10nM dexamethasone (15 days). Human specific hepatic markers albumin, α-feto-protein, insulin-like growth factor-1 (IGF-1), hepatic nuclear factor 4-α (HNF4- α), carbamoyl phosphate synthase 1(CPS1), c-Met and prothrombin expression was tested after differentiation by immunofluorescence. Urea concentrations were measured calorimetrically in the culture media by ELISA. Glycogen storage was visualized by PAS reaction. Immediately after twelve F344-Nude rats were subjected to 90% liver resection, hepatic cells from SHED were transplanted in the spleens (approx. 107 cells /animal) of six of the animals. Twelve F344-Nude rats were subjected to bile duct ligation in order to induce cirrhosis. Sixty days later hepatic cells from SHED were transplanted in the spleens of six animals. Non-transplanted rats from both groups were used as negative control. Animals were sacrificed 40 days after transplantation. Previous human specific hepatic markers were tested by immunohistochemistry. Expression of human-specific albumin in rats' livers was proven by in situ hybridization. Concentrations of human-specific albumin, α-feto-protein and IGF-1 in rats' serum were analyzed respectively by Bromcresol Green, chemiluminescence immunoassay and radioimmunoassay. Results: After in vitro hepatic differentiation α-feto-protein, albumin, IGF-1 and HNF4-α were all shown to be positive by immunofluorescence. Around 90% of cells are deemed as hepatic-like. The concentration of urea in the media increased ($p<0.05$). Glycogen accumulations in the cells' cytoplasm were observed, whereas no accumulation was found in the control. In transplantation study clusters of human-specific hepatic markers expressing tissues, including blood vessels and bile ducts were found in the rats' livers and spleens by immunofluorescence. Human specific albumin, α-feto-protein and IGF-1 were found in rats' serum. Conclusions: Hepatocyte-like cells differentiated in SFM were transplanted into the rats and functioned as human hepatocytes. SHED may therefore be ideal source for cell-therapy of patients requiring liver transplantation.

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Poster Board Number: T-2163

DENTAL PULP REGENERATION: THE ROLE OF PREAMELOBLAST-CONDITIONED MEDIUM ON HUMAN DENTAL PULP STEM CELLS

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Objectives: Regeneration therapy of pulp using human dental pulp stem cells (hDPSCs) has been emerged to maintain the tooth vitality against the conventional endodontic therapy. Preameloblast-conditioned medium (PA-CM) from murine apical bud cells induces the odontogenic differentiation of hDPSCs and promotes dentin formation. The present study aims to evaluate the effect of PA-CM with hDPSCs on pulp regeneration in vivo. Methods: hDPSCs were seeded in the pulp cavity of 5mm-thick human tooth slice with or without PA-CM treatment, and then they were transplanted into immunocompromised mice subcutaneously. Results: In the control group, skeletal muscle with pulp-like tissue was regenerated in the pulp cavity. Reparative dentin-like structure with the entrapped cells was lined on the existing dentin wall. However, in the PA-CM treated group, only pulp-like tissue was generated without muscle and reparative dentin-like structure. Moreover, human odontoblast-like cells exhibited palisade arrangement around the pulp and typical odontoblast processes elongated into the dentinal tubule. Conclusion: These findings suggested that PA-CM induced vascularized pulp-like tissue regeneration of hDPSCs.

Poster Board Number: T-2164

IMMUNOLOGICAL EVALUATION OF HUMAN DENTAL PULP STEM CELLS DIFFERENTIATION WITH EMPHASIZE ON TOLL-LIKE RECEPTOR 4

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Background: Maintaining homeostasis is highly regulated at cellular and molecular level in all tissues including dental pulp. One of the important key regulators of the pulp tissue is human Dental Pulp Stem Cells (hDPSCs), which supports pulp repair / regeneration by their proliferation & differentiation capacity. On the other hand, inflammatory mediators such as cytokines and/or chemokines are pulp tissue molecular regulators. It has been suggested that the inflammatory mediator gradient is controlled by kind of innate immune receptor called Toll-Like Receptor (TLR). TLRs are expressed on various immune cells and even non-immune cells. Interestingly, there are some evidences indicated that TLRs are also expressed in Adult Stem Cells. Among ten functional members of the TLR family in human, TLR4 recognizes very divergent collection of exogenous and endogenous ligands involving in the sterile & pathogen-associated inflammations. It has been recently suggested that TLR4 has multi-functional activities in human mesenchymal stem cell (hMSC) including stem cell proliferation, migration & also differentiation. Here we investigate the comparative expression of TLR4 in hDPSCs and differentiated odontoblasts during the differentiation process. Methods: hDPSCs were enzymatically isolated from wisdom teeth (n=5) and characterized in terms of surface epitopes (hMSC markers CD90/CD73/CD44/CD105 & hematopoietic/endothelial markers CD34/CD45/CD11b/CD31) as well as differentiation capacity into bone, cartilage & adipose cells. Then, hDPSCs were differentiated into odontoblast for 21 days in differentiation medium. Expression of TLR4 gene was analyzed on day 0, 7th, 14th & 21st of odontoblast's differentiation by RT-PCR & Q-PCR. Results: hDPSCs showed MSC phenotype & differentiation capacity into three mesenchyme lineages. Immuno-phenotyping results confirmed the existence of MSC markers and the lack of hematopoietic/endothelial markers. RT-PCR results show the expression of odontoblast differentiation markers dentin matrix acidic phosphoprotein 1(DMP1) & dentin sialophosphoprotein (DSPP) in differentiated cells. The results of RT-PCR indicated the expression of TLR4 gene in hDPSCs on day 0, 7th, 14th & 21st of differentiation. Interestingly, Q-PCR results showed significant increasing expression of TLR4 gene during the differentiation. Conclusion: Immunological evaluation of human adult stem cells has been always considered in stem cell therapy & regenerative medicine. Different expression of TLR4 in differentiated & non-differentiated hDPSCs may shed light on the correlation of innate immunity & differentiation process. Up-regulation of TLR4 may prompt DPSCs to undergo differentiation. Since this difference was significant, less expression of TLR4 may block differentiation process.

Poster Board Number: T-2165

STROMAL PHENOTYPE OF DENTAL FOLLICLE STEM CELLS.

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It has been suggested that stem/progenitor cells exist in dental tissue. This study identified adult mesenchymal stem/stromal cell-like populations in the dental follicle of human impacted third molars.The immunohistochemical analysis, of dental follicle using known stem-cell markers: Cytokeratins (AE1-AE3), Smooth Muscle Actin, Ki-67, CD34, CD44, CD45, CD56, and CD133. A positive reaction for at least one of the markers typical of stromal phenotype (CD56, CD44 and CD271) was observed in seven cases . Interestingly, all positive cases showed coexpression of CD44 and CD56, except for one case which was CD56 positive and CD44 negative. Immunohistochemical reaction was negative in all 27 cases for Ki-67, Cytokeratins, Smooth Muscle Actin, CD34, CD133 and CD45. The association: negative for CD34, CD45, CD133, and positive for CD44, CD56 (markers of a subpopulation of stem cells from bone marrow) suggests these may be quiescent mesenchymal stem cells, a hypothesis supported by the negativity of Ki-67 (proliferative index). Our results are compatible with the identification of immature fibroblast cells with phenotypic features of stromal stem cells in the dental follicle. Acknowledgments: Funding for Stem Cell work was awarded from Ministero dell'Istruzione, dell'Università e della Ricerca -PRIN 2009 (Progetto di Ricerca d'Interesse Nazionale-Grant 2009)

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Poster Board Number: T-2166

EFFECT OF TRANSIENT HYPOXIC STIMULATION ON CULTURED HUMAN PERIODONTAL LIGAMENT DERIVED-CELLS

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Background: Several studies have reported effective cell culture conditions for the isolation and expansion of stem cells that reside in dental pulp and periodontal ligament tissues. A proportion of these stem cells are known to be derived from the cranial neural crest. There are also reports that hypoxic stimulation during culture influences the stem cell function and differentiation. Specifically, hypoxic stimulation was reported to promote the survival rate of neural crest cells, and induced pluripotent stem cell factors in cultured dental pulp-derived cells. Therefore, this "hypoxic treatment" can contribute to isolating more stem cells or to induce the reprogramming of cells with a higher potential for growth and plasticity. However, little is known about the effect of hypoxic treatment on human periodontal ligament-derived cells (PDLCs). Objective: In this study, we investigated whether the surviving human PDLCs cultured under two hypoxic conditions acquired the high growth and plasticity potential. Methods: Human PDLCs were obtained from extracted teeth, digested with collagenase, and the isolated cells sub-cultured until passage 3 for experiments herein. PDLCs were then exposed to either hypoxic (O2<5%) or severe hypoxic/anoxic (O2<0.1%) conditions in low glucose/serum-free media for 6 or 24 hours. Surviving PDLCs were evaluated for the cell viability, alkaline phosphatase (ALP) activity, and gene/protein expressions to determine their stem cell characteristics. Furthermore, multi-lineage differentiation assay was performed to assess their stemness. Results: The severe hypoxic/anoxic treatment (O2<0.1%) led to significantly more cell deaths and reduced ALP levels, when compared with the hypoxic treatment (O2<5%). Expression of Stro-1, CD105 and CD166 was increased in surviving PDLCs of the severe hypoxic treatment group. In particular, stem cell related-genes, such as nanog, oct4, p75 and bmi1, were up-regulated markedly in these surviving cells after 24 hours of severe hypoxic treatment. These data suggest that the transient hypoxic stimulation may influence stemness properties of cultured PDLCs. We are currently investigating the plasticity of these surviving cells in more details, and the usefulness of transient hypoxic stimulation for the expansion of stem cells in PDLCs culture.

Poster Board Number: T-2167

COMPARISON OF PROLIFERATION, EXPANSION AND DIFFERENTIATION INTO SKELETAL CELL LINEAGES OF BLASTAMA CELLS OF RABBIT PINNA AND MURINE BONE MARROW-DERIVED

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Background and purpose: Regeneration could be considered as a new growth of cells similar to lost cells following an injury. This process could be studied in rabbit pinna in which an experimentally-created hole regenerates by forming of a blastema. This tissue consists of a group of undifferentiated cells capable of dividing and differentiating into varying cell types *in vivo*. The exact nature of blastema cells is not well-known hence the purpose of the present study is to isolate and characterize these cells in terms of some well-known criteria for mesenchymal stem cells including colonogenic activity and differentiation potential into skeletal cell lineages and compare this properties with Bone marrow mesenchymal stem cells. In this study culture requirement of the isolated cells also explored and compared between two kind of cells. Methodes: Blastema and MSC cells were collected from Five News land white male rabbit with 3-6 month ages and the culture was expanded. The isolated cells were then compared in terms of in vitro differentiation capacity and some growth and proliferation properties such as colony forming assay, growth curve and concentrations of fetal bovine serum (FBS) at varying initiating cell density Results:Two kind of cells appeared to be able to differentiate into bone, cartilage and adipose cell lineage but Blastema cells were observed to be more proliferative than BM-cells. also at growth characteristics they are kind of cells with high proliferate and expansion than MSC and they have ability to form colonies and can expand with less FBS concentration in medium. Conclusions: Taken together it could be concluded that blastema from rabbit ear contains a population of fibroblastic cells much similar in characteristic to Bone marrow mesenchymal stem cells even housed MSCs with a relatively higher proliferation property than BM-derived MSCs and a typical tripotent differentiation potential comparable with marrow MSCs, hence it would be as an appropriate source of MSCs for use in regenerative medicine related studies

Poster Board Number: T-2168

DIFFERENTIAL IMMUNOSUPPRESSIVE PROPERTIES OF MOUSE MSCS ON TH1 CELLS

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In recent years, many authors have described that Mesenchymal Stem Cells (MSCs) are able to suppress the proliferation of T-lymphocytes although the mechanism by which MSCs exert this effect is still unclear. The aim of our study was to determine if MSCs were able to suppress the proliferation and the differentiation of Th1 cells *in vitro* and to evaluate dependency on T-cell activation state, cell-to-cell contact and Tcell:MSCs ratios. Mesenchymal stem cells were obtained from mice bone marrow and characterized by their surface antigen expression pattern and their capability of multilineage differentiation. T-CD4+ cells were obtained from mice splenocytes and differentiated into Th1 cells. MSCs were added to Th1 cultures either at early (day 0) or late (day 2 and 4) time points in the presence or absence of a transwell system. After 6 days of co-culture, Th1 populations were measured by flow cytometry using intracellular IFN-gamma. We demonstrate that MSCs effectively suppress Th1 cells in spite of their activation state and T-cell:MSC ratios. MSCs are able to diminish IFN-gamma secretion up to an 85% ($p<0.5$) when they are added early on cultures and 26% ($p<0.5$) when added at later time points. Previous reports have shown that MSCs IL6-/- are unable to suppress T cell activation and proliferation, therefore we investigated if this factor was responsible for Th1 suppression. Surprisingly, and in contrast to what was observed on non-differentiated T-cell, MSCs IL-6-/- where also able to suppress Th1 cells. Importantly, the mechanisms used by MSCs to suppress different subtypes of T-cells are different since differentiated Th1 cells are suppressed in the absence of IL-6 while non-differentiated T cells are not. Our findings suggest that the mechanisms of MSC-mediated immune suppression are more complex and dynamic

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than previously thought, which must be further explored due to the potential use of MSCs as a treatment for autoimmune diseases.

Poster Board Number: T-2169

TOWARD UNDERSTANDING THE BALANCE BETWEEN OSTEOBLASTOGENESIS AND ADIPOGENESIS IN BONE MARROW MICROENVIRONMENT.

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The majority of conditions associated with bone loss, including osteoporosis were shown to be accompanied by increased marrow adiposity due to shifting in the balance between osteoblast and adipocyte differentiation of bone marrow skeletal stem cells (MSC). However, little is known about how the balance between osteogenesis and adipogenesis is regulated in the bone marrow microenvironment. Recently, we proposed a novel molecular model for lineage fate determination of MSC that is regulated through the cross-talk between two unique populations of pre-committed osteoblasts (MSC-OB) and adipocytes (MSC-Adipo) rather than through changes in the differentiation pathway of multipotent MSC (Taipaleenmäki H, Abdallah BM . Exp Cell Res. 2011; S. Post, B.M. Abdallah. Bone. 2008). Thus, we aimed in this study to identify novel secreted factors that mediating the cross-talk between osteoblasts and adipocytes in bone marrow. For that purpose, we used a combination of microarray (GeneChip® MG430A 2.0 Array) and mass spectrometry-based proteomics (secretome) to identify secreted proteins by MSC-OB (only can differentiate into osteoblast) versus MSC-Adipo (only can differentiate into adipocyte). Transcriptome and secretome profiles of these two cell lines were compared using different criteria including bioinformatics, function annotation analysis, gene expression analysis and extensive literature survey. After excluding all previously studied factors in MSC differentiation, we identified 7 novel secreted proteins (three proteins by MSC-Adipo and other 4 by MSC-Adipo). Studying the molecular function of these proteins in MSC cells revealed a selection of 3 secreted factors with the potential to control the commitment of MSC into either osteoblast or adipocyte lineage. In conclusion, cross-talk exists between different populations of pre-committed osteoblast and adipocyte cells in bone marrow to regulate differentiation of multipotent MSC through novel secreted factors.

Poster Board Number: T-2170

DIFFERENTIATION OF MOUSE UMBILICAL CORD DERIVED MESENCHYMAL STEM CELLS *IN VITRO* INTO HEPATOCYTE LIKE CELLS

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Objective: The purpose of this study is to differentiate mesenchymal stem cells into hepatocyte-like cells by adding liver extract on mesenchymal stem cells (MSCs), which manifest the morphology of hepatocyte cells. To prove this similarity, we use Periodic Acid-Schiff test. Methods: MSCs were isolated from NMRI mice on days 14-17 and cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS (Fetal Bovine Serum).We investigated the differentiation potential of mouse mesenchymal stem cells into hepatocytes in vitro by using liver extract every 4 days. We cultured mesenchymal stem cells without additional factors or late differential markers of hepatic development. Periodic Acid Schiff test was used to indicate this differentiation. Results: Our results have shown that mesenchymal stem cells were changed to hepatocyte-like cells after 30-35 days. These cells have polygonal morphology of hepatocyte-like cells with 1 or 2 obvious nuclei Periodic Acid -Schiff (PAS) test was used for detecting the amount of glycogen synthesis in differentiated hepatocyte-like cells. The red colour confirmed this differentiation. Conclusion: The proposed procedure for hepatocyte cells differentiation by using mesenchymal stem cells from mouse umbilical cord and diluted liver extract demonstrates that mouse mesenchymal stem cells can change to hepatocyte-like cells with liver extract treatment. Keywords: Mesenchymal Stem Cells (MSCs), in vitro, Differentiation, liver extract, Periodic Acid-Schiff, hepatocyte-like cells 1

Poster Board Number: T-2171

PROTEOMIC ANALYSIS OF INTEGRIN-ASSOCIATED COMPLEXES FROM MULTIPOTENT AND EARLY DIFFERENTIATED MESENCHYMAL STEM CELLS

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Stem cell differentiation is an important process in maintaining bone homeostasis. Bone marrow mesenchymal stem cells (MSCs) are multipotent cells with the potential to differentiate into adipocytes and osteocytes and are responsible for maintaining bone homeostasis. It has been shown that a shift of MSC differentiation toward adipogenesis leads to reduced osteogenesis. The process that drives MSCs into an adipogenic versus an osteogenic lineage in the bone marrow is poorly understood. Numerous factors in the MSC microenvironment have been identified to be involved in MSC function, such as cell-extracellular matrix (ECM) adhesion, cell-cell interactions and soluble factors. Adhesion of cells to the ECM is mediated by integrin adhesion receptors. Following adhesion, integrins cluster at the cell membrane, and protein complexes called focal adhesions (FAs) build up inside the cells. FA assembly is likely to induce signalling events in cells and affects cell fate. In this study, a proteomics-based system has been developed to isolate, identify and quantify integrin-associated complexes in undifferentiated MSCs and MSCs induced to an adipocyte lineage (short-term induction, 3 hours). The aim of this study was to compare the composition of FAs and the abundance of their components in these two cell lines to identify proteins that may be involved in MSC differentiation to an adipocyte lineage. MSCs were allowed to adhere to surfaces coated with defined ECM proteins. Following incubation, equal populations of MSCs were subjected to two different treatments for 3 hours: 1) the adipogenic induction medium; or 2) adipogenic maintenance medium (control). Ligand-induced integrin-associated complexes were stabilized using crosslinker. Cells were lysed, and isolated complexes were subjected to downstream proteomic analysis. Mass spectrometry (MS) analysis showed the enrichment of key proteins, such as integrin β1, integrin α5, vinculin, talin, α-actinin-4, zyxin, tensin, filamins, kindlin-2, integrin-linked kinase (ILK) and the actin-binding proteins PDZ and LIM domain 1 and 7 (Pdlim1 and Pdlim7) in ECM-bound complexes from non-induced and induced MSCs compared to complexes isolated from a non-ECM ligand (control). These data were validated by immunoblotting. Furthermore, the comparison of spectral counts (used as a measure of protein abundance) between the integrin-associated complexes in non-induced and induced MSCs showed a remarkable difference in protein abundance in these two cell lines. These data demonstrate that integrin adhesion complexes can be isolated in a reproducible manner from non-

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induced and induced MSCs in a way suitable for MS analysis. They also indicate that the early induction of an undifferentiated cell line (MSCs) into a certain lineage (adipogenesis) increased the abundance of key FA proteins, specifically in integrin-associated complexes induced by ECM ligands. Future work will be directed towards obtaining further in-depth MS analyses, which, followed by bioinformatics evaluation, will implicate molecules involved in the integrin-mediated regulation of stem cell maintenance and differentiation in non-induced and induced MSCs.

Poster Board Number: T-2172

EVALUATING THE EFFICACY OF ENDOTHELIAL CELLS DIFFERENTIATED FROM ADIPOSE DERIVED MESENCHYMAL STEM CELLS FOR *IN VITRO* INTIMA CONSTRUCTION

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Adipose tissue is an attractive candidate to become a major stem cell source for regenerative medicine and tissue engineering applications. Adipose-derived mesenchymal stem cells (AdMSCs) possess multilineage differentiation potential. Differentiation into mesodermal cell types, i.e. into adipocytes, chondrocytes, osteoblasts, and myocytes has been shown in several studies. They also can be induced to differentiate into ecto and endodermal origin, e.g. hepatocytes, pancreatic islet cells, neural cells, epithelial cells, and endothelial cells (ECs). The objective of this study was to investigate the differentiation capacity of human (h) AdMSCs into vascular endothelial-like cells, and to evaluate the efficiency of their use for *in vitro* construction of a functional tunica intima layer into an engineered-vascular scaffold. Methods. hAdMSCs were isolated from human lipoaspirates, cultured, then immunophenotypically characterized by FACS and by their tri-lineage differentiation potential. Passages 4-6 were used in all experiments. AdMSCs were seeded on collagen type IV-coated surfaces and cultured for 7 and 14 days in the EC-GM containing FGF2, VEGF, heparin, IGF-I, EGF, hydrocortisone, and ascorbic acid. hAdMSCs were also seeded inside a nanofibrous tubular polymer scaffold and differentiated into ECs either in static or flow conditions. As positive control, human internal mammary artery endothelial cells (hIMAECs) were isolated and expanded on bovine skin gelatin-coated tissue culture plates in EC proliferative medium (EC-PM); hAdMSCs cultured in standard medium without differentiation inducers served as the negative control in all experiments. To evaluate EC characteristics, immunohistochemistry studies were performed using antibodies against six EC-specific proteins. Additionally, acetylated-low density lipoprotein (ac-LDL) uptake and expression of endothelial cell nitric oxide synthase (eNOS) were determinated for endothelial cell function analysis. Results: Human AdMSCs had CD 90+, CD105+, CD73+, CD29+, CD44+, CD34-, CD45-, and CD133- immunophenotype and demonstrated tri-lineage differentiation potential. Immunohistochemistry demonstrated that hAdMSCs on nanofibrous tubular construct differentiated into endothelial-like cells, and expressed CD31 (PECAM-1), CD34, von Willebrand factor (vWF), VE- cadherin (CD144), tie-2 and flk-1 (VEGFR2) at day 7. Overall, the expression levels of these endothelial specific proteins significantly increased after 14 days which were comparable with that of the hIMAECs. While the expressions of eNOS and ac-LDL uptake were found to be quite low in static culture, a positive influence was observed under *in vitro* biomimetic shear conditions. Conclusion. As the conclusion, human adipose mesenchymal stem cells can be differentiated into functional vascular endothelial-like cells inside a nanofibrous tubular scaffold under flow conditions that express EC-specific proteins. Findings support the notion that the tunica intima could be constructed *in vitro* using ECs derived from hAdMSCs. Currently, we are testing the possibility of increasing expression levels of eNOS and ac-LDL uptake by constructing a media layer containing smooth muscle cells in close contact with the neointima layer inside a a real time computer-controlled bioreactor system.

Poster Board Number: T-2173

ROLE OF POLYCOMB PROTEINS RING1A/B IN MAINTENANCE OF THE MESENCHYMAL STEM CELL NICHE AND REGULATION OF CELL DIFFERENTIATION

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Adult mesenchymal stem cells (MSCs) provide a source for tissue growth and repair. Unlike human teeth, mouse incisors grow continuously, which is achieved by stem cells residing at their apical end. Compared to the dental epithelial stem cell niche that is well characterized, little is known about the dental mesenchymal stem cell niche. We recently found polycomb repressive complex1 (PRC1) Ring1a/b and their complex proteins strongly expressed in the mesenchymal area of the apical end of incisors during mouse incisor development. In Ring1a/b double knock-out (DKO) mice, we found a disruption of enamel and dentin formation in continuously growing incisors, and a dramatically reduced proliferation of the apical mesenchymal and cervical loop epithelium in comparison to Ring1a-/-;Ring1bfl/fl cre- incisors. The MSC marker, Thy1 is expressed in a small group of slow proliferating cells at the most apical end of mouse incisors between the cervical loop epithelium and close to the Ring1a/b expression area. Genetic lineage tracing of these cells using Thy1-cre mice shows that they form all mesenchymal cells of the growing tooth. Microarray analysis identifies down-regulation of Wnt3a and Thy1 expression in Ring1a-/-;Ring1bcko/cko mice, whereas MSC differentiation markers Bmp6, Pparg and Runx2 are up-regulated in the Ring1a/b DKO mice incisors. Our data shows that PRC1+ve cells are highly proliferative and located close to slowly proliferating Thy1+ve MSCs. This suggests that the PRC1+ve cells are transit amplifying cells and that PRC1 proteins are required not only for differentiation of these cells but also for a role for these cells on the MSCs.

Poster Board Number: T-2174

GLUTATHIONE DEPENDENT OSTEOGENIC DIFFERENTIATION OF SKIN MESENCHYMAL PROGENITORS OCCURS THROUGH MAPK SIGNALING

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Mesenchymal stem cells (MSCs) are mesodermal precursors located at the dermis, capable of osteogenesis. Increased reactive oxygen species (ROS) levels have been implicated in delayed bone development in mouse embryo and hFOB pre-osteoblasts, but little is known about the impact of ROS in stem cell osteogenic differentiation. We induced murine skin MSCs to osteocyte differentiation for 7, 14 and 21 days (terminal differentiation) and in short periods

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0.1, 0.5, 1, 2 6 and 24h (commitment). These cells were successfully differentiated, producing calcium/phosphate matrix (Alizarin-Red, Von-Kossa stain) and increased mRNA levels of alkaline-phosphatase, osteopontin and osterix. We then analyzed the contribution of the canonical and glutathione related antioxidant system along the differentiation through qRT-PCR, WB and enzymatic activity. The canonical antioxidant pathway apparently does not play an important role in this model, since no significant differences in the levels of catalase and Cu/ZnSOD were found, except for MnSOD (n=3, p≥0.05). For the glutathione-dependent system, we found increased levels of glutathione peroxidase isoforms and glutathione reductase. In agreement with these data, the thioredoxin/peroxiredoxin system is also upregulated (n=3, p≤0.001), and the balance between GSH/GSSG altered (HPLC), suggesting a role for the glutathione-dependent system in the differentiation process. We then set out to evaluate which pathways could perform this redox-regulated signaling. The kinases p38, ERK1/2 and JNK were differentially phosphorylated during commitment of differentiation and displayed differential patterns when the differentiation was performed in the absence of GSH (depletion through BSO) or in a more reduced-versus-oxidized intracellular environment (DTT/H2O2). Also, the expression of some of the glutathione-related enzymes was abrogated when these MAPKs were pharmacologically inhibited. In brief, the relative levels of GSH/GSSG and related antioxidant system are differentially regulated through a MAPK axis during skin MSC differentiation leading us to propose a central role for this thiol in the maintenance/commitment of stem cells during osteogenesis.

Poster Board Number: T-2175

IL1BETA IS A POTENTIAL STROMAL GROWTH FACTOR

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Here we demonstrate, for the first time, the ability of IL1-beta - a well known proinflammatory cytokine, - to stimulate the development of hematopoietic microenvironment *in vivo*. Mesenchymal stem cells (MSCs) are capable to transfer hematopoietic microenvironment. After implantation of femur bone marrow plug under the renal capsule of syngeneic recipients the ectopic hematopoietic foci are formed. Stromal cells in such foci derived from donor MSCs while hematopoietic cells have recipient's origin. The size of the foci formed (estimated by nucleated cell number) is proportional to the femur equivalent transplanted. This can be used for semi-quantitative determination of MSCs number. In this work we show that IL1-beta injections during ectopic foci formation lead to the enlargement of hematopoietic territory within such foci. Methods: The bone marrow plug was implanted under the renal capsule of syngeneic (C57Bl x CBA) F1 recipients. Six weeks later the ectopic foci of hematopoiesis were analyzed. IL1-beta have been injected in transplanted mice intraperitoneally and subcutaneously in dose 25, 100, 200 and 400 pg per mouse once a day for the first 3 weeks (15 injections totally) after implantation of bone marrow. Each group consisted of 6 transplants. The size of each focus was determined 6 weeks after implantation by nucleated cell count and by weighing of bone shells. The concentration of IL1-beta in the serum of control and injected animals was determined by ELISA at the end of the experiment. Results: Experimental animals did not demonstrate any signs of toxicity. The size of ectopic hematopoietic foci in control group was 6.5 ± 0.5·10^6 cells while it was larger in every injected groups - 7.2 ± 0.8, 10.8 ± 0.5, 10.6, and 7.7 ± 1.2·10^6 of nucleated cells for 25, 100, 200 and 400 pg of IL1-beta, respectively. In the group which was injected with 100 pg of IL1-beta the size of hematopoietic foci was 1.7 times larger than in control group and that was statistically significant (P = 0.001). Thus the effect was dose-dependent with the optimal dose of IL1-beta for stimulating hematopoietic environment being 100 pg. The weight of bone shells was 2.8 ± 0.1 mg for control group, 3.7 ± 0.1, 4.8 ± 0.1, 3.5 and 2.3 ± 0.3 mg for each experimental group, respectively. These results demonstrate that injecting 100 pg of IL1-beta lead to the formation of larger foci of hematopoiesis with both higher amount of nucleated cells and larger bone shells. The actual size of hematopoietic territory may depend not only on the number of MSCs but also on more mature mesenchymal progenitor cells. These cells, as they differentiate, create new bone and stroma thus providing new niches for hematopoietic cells which seem to be analogous to the osteoblastic and vascular niches in the bone marrow. The results obtained indicate that IL1-beta possesses new unexpected property - to stimulate the formation of hematopoietic microenvironment and bone tissue *in vivo* by presumably acting on mesenchymal progenitor cells while one can not exclude its effect on MSCs. Such novel stromal growth factor may be useful for treatment of several diseases affecting either hematopoietic microenvironment or bone defects. Properties of IL-1beta as stromal growth factor require further detailed investigations. One of the most interesting questions is to determine target cells in the hierarchy of mesenchimal stem cells for this cytokine which is still obscure.

Poster Board Number: T-2176

DIABETES CAUSES ABNORMALITIES IN THE BONE MARROW NICHE BETWEEN OSTEOBLASTIC NICHE CELLS AND LONG-TERM RECONSTITUTING HEMATOPOIETIC STEM CELLS

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Interaction between hematopoietic stem cells and bone marrow niche cells through chemokines and their receptors as well as cell adhesion molecules maintains the balance in hematopoietic stem cells (HSCs) between cell division/proliferation and quiescence. Although it is well accepted that diabetes induces functional abnormality in hematopoietic stem/progenitor cell mobilization and proliferation, the interaction between bone marrow niche cells and their partner long-term reconstituting HSCs (LT-HSCs) has not been investigated. Here we isolated osteoblastic niche cells (Lin– OPN+) and LT-HSCs (Lin– Sca-1+ c-Kit+ CD135– CD34–) from STZ-diabetic mice and examined the expression of molecules essential to maintain bone marrow niche functions. To mimic the microenvironment in the bone marrow niche, we established *in vitro* co-culture between LT-HSCs and osteoblastic niche cells at normoglycemic or high glucose conditions. In diabetic mice osteoblastic niche cells and LT-HSCs were decreased in number, and expressions of N-cadherin and β-catenin on LT-HSCs as well as β1-integrin on osteoblastic niche cells were significantly reduced compared to non-diabetic mice. Expressions of CXCL12 and angiopoietin-1 on the osteoblastic niche, and receptor tyrosine kinase Tie2 and receptor for Wnt signals LRP6 on LT-HSCs were significantly reduced in diabetic mice. *In vitro* co-culture experiment, osteoblastic niche cells derived from diabetic mice can lodge onto the bottom under high glucose condition, in the contrary those derived from nondiabetic mice can lodge onto the bottom under normal glucose condition. LT-HSCs were maintained in contact to osteoblastic niche cells and kept stemness for 7 days. Expressions of N-cadherin, β-catenin and Tie2 on LT-SHCs derived from nondiabetic mice were reduced when

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exposed to diabetic osteoblastic niche cells. Conversely, reduced expression in these molecules on diabetic LT-HSCs was reversed into normal levels when exposed to nondiabetic osteoblastic niche cells in co-culture system. By these experiments we succeeded to reverse diabetes-induced abnormality in LT-HSCs by replacement of osteoblastic niche cells and this may provide the future perspectives for treatment of diabetes-induced complications in hematopoietic cells.

Poster Board Number: T-2177

MIR-124 PROMOTES NEURONAL DIFFERENTIATION OF ADULT MESENCHYMAL STEM CELLS BY TARGETING THE ANTI-NEURAL REST/SCP1 PATHWAY AND SOX9

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Adult mesenchymal stem cells (MSCs) isolated from the bone-marrow and adipose tissue exhibit self-renewal and differentiation into multiple lineages including bone, cartilage and adipose cells. Recent studies suggest that these cells can also acquire neuronal phenotypes under certain growth conditions. MicroRNAs (miRs) are small non-coding RNAs that act as important post transcriptional regulators by either inducing their target mRNA cleavage or by translational repression. Specific miRs have been implicated in various differentiation processes and in cell transformation and their ability to regulate large numbers of target genes implicate them as important regulators of stem cell differentiation. In this study we examined the role of the neuronal-related miR-124 in the neuronal differentiation of adult bone-marrow and adipose-derived MSCs. miR-124 significantly decreased cell proliferation, induced a neuronal-like morphology and increased the expression of voltage-dependent sodium channels and the neuronal markers Tuj1 and NeuN. On further characterization, we found that the MSCs preferentially expressed tyrosine hydroxylase and additional dopaminergic markers. miR-124 decreased the expression of its potential targets, STAT3, CDK6, ROCK, Sox9, Jagged 1, PTBP1 and the phosphatase SCP1 which exhibits anti-neural function. miR-124 directly targeted the Sox9 and SCP1- 3′ UTRs and overexpression of SCP1 and Sox9 partially abolished the proneuronal effect of miR-124 in the MSCs, suggesting that additional pathways may be involved in this effect. We conclude that miR124 promotes the neuronal differentiation of MSCs by targeting multiple signaling pathways and could be employed to generate a potential cellular source for cell replacement therapy in various neurodegenerative disorders.

Poster Board Number: T-2178

MICRORNA-145 REGULATES OSTEOGENIC LINEAGE DIFFERENTIATION BY TARGETING TRANSCRIPTION FACTOR CBFB

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Bone marrow derived mesenchymal stem cells are self-renewing multipotent cells that can differentiate along several lineages, including the osteoblast lineage, in response to diverse factors. Understanding the regulatory mechanism of osteoblast differentiation is a prerequisite for treatment of bone loss diseases such as osteoporosis. MicroRNAs (miRNAs) are small non-coding RNAs that significantly inhibit the translation of target genes. miRNAs have emerged as important regulators in various developmental, physiological and pathological conditions. Previous reports implicated some miRNAs in the differentiation of osteoblasts and osteoclasts. However, their importance in the regulation of osteogenic differentiations remains to be elusive. In the present study, we screened for differentially expressed miRNAs during osteoblast differentiation of MC3T3-E1 cells and identified miR-145 as a potential regulator of osteogenesis. Overexpression of miR-145 in MC3T3-E1 cells inhibits ALP activity and osteoblast specific gene expression. To understand the molecular mechanisms that underlie the miR-145 mediated regulation, we searched for potential targets of miR-145 implicated in osteoblast differentiation using in silico analysis. Among the predicted targets, we identified core binding factor beta (Cbfb), a heterodimeric partner for Runt-related transcription factor 2 (Runx2) in which the essential transcription factor in skeletal development by regulating osteoblast differentiation. To determine whether miR-145 inhibits Cbfb gene expression by binding to the predicted target site in the 3′-UTR, we used a luciferase reporter analysis. Co-transfection of Cbfb 3′-UTR containing reporter with miR-145 resulted in significantly decreased luciferase activity compared with control. Furthermore, ectopic expression of miR-145 down-regulated endogenous Cbfb mRNA and protein expression. Taken together, these results suggested that miR-145 regulates osteogenic differentiation at least in part by inhibiting the Cbfb expression. Our finding suggested that pharmacological inhibition of miR-145 may lead to the therapeutic strategy for bone degenerative diseases such as osteoporosis.

Poster Board Number: T-2179

TAUROURSODEOXYCHOLIC ACID ATTENUATES ADIPOGENESIS OF ADIPOSE-DERIVED STROMAL CELLS BY DECREASING ENDOPLASMIC RETICULUM STRESS

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Human adipose-derived stromal cells (ASCs) are a heterogeneous group of multipotent progenitor cells that are able to differentiate into various types of cells such as adipocyte, chondrocyte, osteoblast etc. Therefore, it is critical to appropriately induce ASC differentiation with high efficiency toward target cells. Tauroursodeoxycholic acid (TUDCA) is a commercially available bile acid derivative to treat cholestatic liver disease and cholelithiasis. Recently, it has been reported that TUDCA not only has the ability to decrease endoplasmic recticulum (ER) stress, also plays a role as leptin-sensi-

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tizing agents in obese mice and human. In this study, we examined whether TUDCA affects adipogenic, chondrogenic and osteogenic differentiation of ASCs and then evaluated through RT-PCR, histological staining and so on. TUDCA treatment significantly decreased ER stress of ASCs resulting in decrease of adipogenic differentiation of ASCs. However, TUDCA treatment did not affect osteogenic and chondrogenic differentiation. Thus, TUDCA would be a useful ingredient for cartilage and bone tissue regeneration of stem cells by inhibiting adipogenic differentiation. Keywords: Tauroursodeoxycholic acid, Adipose-derived stromal cells, endoplasmic reticulum stress, Adipogenic differentiation.

Poster Board Number: T-2180

EXPRESSION OF HOX GENES IN THE BONE MARROW DERIVED MESENCYHMAL STROMAL/ STEM CELLS FROM FANCONI ANEMIA PATIENTS CHANGES FOLLOWING BONE MARROW TRANSPLANTATION

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HOX transcription factors are encoded by thirty-nine genes clustered in four different chromosomes in mammalian and designated as HoxA, HoxB, HoxC, HoxD. HOX code provides important intrinsic regulatory program for cell fate. Whether HOX code has a role as an extrinsic factor and how HOX code changes in hematopoetic niche in disease states are interests of our research group. In this study, we investigated HOXOME profile of bone marrow derived mesenchymal stromal cells (MSC) from Fanconi anemia patients before and after bone marrow transplantation. Fanconi anemia (FA) is a cancer predisposition disorder characterized by progressive bone marrow failure. Bone marrow derived mesenchymal stromal cells (MSC)from donors and FA patients (before and after transplantation) were expanded and characterized for their stromal cell surface markers and differentiation capacity to adipocytes and osteoblasts. HOXOME profile was determined by real-time RT-PCR using Taqman probes. Expression of Hox genes in the third passage of MSCs mostly showed decrease in FA patients comparing to donors. Among down-regulated genes, HoxA5, HoxA6 and HoxA10 were obtained to be rescued after the bone marrow transplantation.

Poster Board Number: T-2181

INDUCTION OF NEURAL PHENOTYPE IN EMBRYONIC STEM CELL-DERIVED MESENCHYMAL PROGENITORS BY RHOA-KINASE INHIBITION

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Current advances in stem cell biology have brought much hope for therapy of neuro-degenerative diseases. However, neural stem cells (NSCs) are rare adult stem cells, and the use of non-NSCs requires efficient and high-yielding lineage-specific differentiation prior to transplantation for efficacy. We report on the efficient differentiation of embryonic stem cell-derived mesenchymal progenitors (EMPs) into a neural phenotype with use of Y-27632, a clinically compliant small molecular inhibitor of Rho kinase (ROCK). EMPs are a population of mesenchymal progenitors derived from human embryonic stem cells (hESCs) which are capable of multilineage differentiation but do not form teratomas. These early mesenchymal progenitors are an ideal stem cell source since they are non-tumorigenic and immunomodulatory¡Xsimilar to adult bone marrow mesenchymal stem cells¡Xbut do not easily senesce after *in vitro* culture, and are able to be regenerated indefinitely along from ESCs and other pluripotent stem cells including induced pluripotent stem cells. We treated EMPs with Y-27632 and investigated for differentiation capacity and neural lineages by gene expression, and protein expression. Y-27632 induced EMPs into a neural-like morphology, with rapid development of cell extensions and processes within 24 hours. Y-27632-treated EMPs express several neural lineage genes at the RNA and protein level, such as Nestin, MAP2, Tuj1 and GFAP. EMPs can differentiate into a neural phenotype via inhibition of RhoA/ROCK pathway by Y-27632. Further investigation of the detail mechanisms of neural differentiation is needed to improve the efficiency of differentiation for clinical therapeutic use.

Poster Board Number: T-2183

STEM CELL DIFFERENTIATION CAN BE DIRECTED BY SCAFFOLDS WITH ADHESIVE DOMAINS

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Human mesenchymal stem cell (hMSC) differentiation has been examined extensively in two dimensional environments, but most materials examined to date do not mimic the adhesive heterogeneity of native extracellular matrix; as such, it is unclear if the spatial arrangement and size of these adhesives regions play any role in guiding stem cell fate. Here, we report on a process where porous foams are fabricated by high internal phase emulsion (HIPE) templating using amphiphilic copolymers. The different copolymers assemble at the oil-water interface and undergo confined phase separation. This creates foams' surface topology with nanoscopic domains of cell inert and active chemistries detected by the chemical force spectroscopy based on the atomic force microscopy. These results qualitatively resemble native matrix, and results show localized protein deposition in domains of active chemistry. hMSC adhesion then occurs through specific copolymer domains. Interestingly, without the induction media, hMSCs are likely to express linage specification corresponding to these 'patchy' matrices, and their expression depends on stem cell origin: marrow-derived and mesenchymal progenitor cells exhibit fundamentally different differentiation patterns, adipo- and osteo-genic, respectively. Together these data implicate adhesion as a complex regulator of cell fate.

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EVALUATION OF HEPATOGENIC DIFFERENTIATION POTENTIAL OF MENSTRUAL BLOOD DERIVED STEM CELLS

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Introduction: Recently, menstrual blood has been identified as an easily accessible and renewable stem cell source. However, the challenge remains to develop robust protocols to generate different lineages such as hepatocyte from MenSCs. In this study, hepatogenic differentiation capacity of menstrual blood derived stem cells (MenSCs) was investigated in presence of different combination of growth factors and cytokines. Methods: MenSCs were isolated of menstrual blood samples by discontinuous density gradient centrifugation and plastic adherence. After karyotypic and immunophenotypic analysis, differentiation ability of cultured cells into hepatocyte using three-stage methods developed by different combination of hepatocyte growth factor (HGF), oncostatin M (OSM), epidermal growth factor, basic fibroblast growth factor, insulin-like growth factor, dexamethasone and nicotinamide was evaluated. Results: Flow cytometric analysis illustrated that MenSCs can typically express CD29, CD44, CD73, CD105, OCT-4 and CD10 while lacking CD34, CD38, CD133 and CD45. Immunofluorescence staining and enzyme-linked immunosorbent assay showed that unlike undifferentiated MenSCs, albumin accumulation and secretion was significantly detectable in differentiated cells in different combination of growth factors and cytokines. However, positivity rate of albumin expression was dependent to concentration of HGF and OSM. The cytochemical observations were corroborated with the extent of mRNA expression of albumin and another hepatic specific markers such as cytokeratin-18, cytokeratin-19 and alpha-fetoprotein evaluated by real-time PCR analysis. Conclusion: The evidence presented here introduces MenSCs as unique population cells with great differentiation ability into hepatocytes-like cells.

Poster Board Number: T-2185

A NOVEL BONE MARROW-DERIVED, VESSEL-RESIDENT CALCIFYING PROGENITOR CELL POSSESSES BI-DIRECTIONAL (OSTEOBLASTIC/OSTEOCLASTIC) DIFFERENTIATION POTENTIALS.

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Background: Vascular calcification is an advanced feature of atherosclerosis. But, currently no effective therapy is available. To modulate or even reverse the process of calcification, we aimed to identify calcifying progenitor cells and investigated calcifying/decalcifying potentials of these cells. Methods and Results: Cells from aortas of mice were sorted into 4 groups using Sca-1 and PDGFRα markers. Sca-1+ (Sca-1+/PDGFRα+ and Sca-1+/PDGFRα-) progenitor cells showed greater osteoblastic differentiation potentials than Sca-1- (Sca-1-/PDGFRα+ and Sca-1-/PDGFRα-) cells. Interestingly, among Sca-1+ progenitor populations, Sca-1+/PDGFRα- progenitor cells possess bi-directional differentiation potentials towards both osteoblastic and osteoclastic lineages, whereas Sca-1+/PDGFRα+ progenitor cells differentiate into the osteoblastic lineage unidirectionally. When treated with PPARγ agonist, Sca-1+/PDGFRα- cells preferentially differentiated to osteoclasts. Sca-1+ calcifying progenitor cells in the artery originated from bone marrow (BM) and can be clonally expanded. Vessel-resident BM-derived Sca-1+ calcifying progenitor cells showed non-hematopoietic, mesenchymal characteristics. In order to test their capability to modulate in vivo calcification, we next preformed two kinds of experiments, ectopic and atherosclerotic calcification. CT scoring revealed that Sca-1+ progenitor cells increased the volume and calcium score of ectopic calcification. But, Sca-1+/PDGFRα- cells when treated with PPARγ agonist decreased the bone formation. Systemic infusion of Sca-1+/PDGFRα- cells into Apoe-/- mice increased calcified atherosclerotic plaques. However, Sca-1+/PDGFRα- cells with PPARγ activation markedly deceased plaques, indicating bi-directional fate in vivo. Conclusions: These findings suggest that a subtype of BM-derived and vessel-resident progenitor cells offer a therapeutic target for the treatment of vascular calcification, and the PPARγ activation may be an option to reverse calcification

Poster Board Number: T-2186

CHONDROGENIC AND ADIPOGENIC DIFFERENTIATION POTENTIAL OF MENSTRUAL BLOOD- VERSUS BONE MARROW-DERIVED STEM CELLS

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Aim: Researchers discovered that menstrual blood is easily accessible, refreshing and inexpensive source of stem cells. The aim of this study was to investigate chondrogenic and adipogenic differentiation potential of the menstrual blood derived stem cells (MenSCs) compared to that of bone marrow derived stem cells (BMSCs). Methods: MenSCs from menstrual blood and BMSCs from human bone marrow were separated under standard conditions in Ficoll gradient. After characterization of isolated cells, chondrogenic and adipogenic differentiation potential of MenSCs compared to that of BMSCs was evaluated by cytochemical staining and molecular experiments. Results: MenSCs were strongly positive for mesenchymal and negative for hematopoietic stem cell markers in a similar manner to BMSCs. In contrary to BMSCs, MenSCs exhibited marked expression of OCT4 and higher proliferative capacity. Differentiated MenSCs into chondrocytes in a similar pattern with BMSCs had strong immunoreactivity with monoclonal antibodiy against Collagen type 2 and accumulation of proteoglycan in differentiated MenSCs was comparable with that of differentiated BMSCs. However, the mRNA expression pattern of chondrogenic markers such as Collagen 2A1, Collagen 9A1 and SOX9 in MenSCs was different with that of BMSCs as judged by RT-PCR. Adipogenesis as judged by oil red O- staining was more pronounced in differentiated BMSCs compared to differentiated MenSCs. Conclusion: MenSCs are unique stem cell population with higher proliferation, comparable chondrogenic and lower adipogenic differentiation ability compared to BMSCs. Much quantitative studies at molecular level will help us to find out the reason of obtained differences between MenSCs and BMSCs.

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Poster Board Number: T-2187

IDENTIFICATION OF DJPIWIB TARGETS TO CLARIFY THE MOLECULAR FUNCTION OF DJPIWIB IN THE PLURIPOTENCY OF PLANARIAN ADULT SOMATIC PLURIPOTENT STEM CELLS

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The freshwater planarian Dugesia japonica has high regenerative ability, by which it can regenerate complete individuals from even tiny fragments from almost all parts of its body. This ability depends on adult somatic pluripotent stem cells called "neoblasts". Interestingly, these stem cells have some morphological and molecular features similar to those of germ-line cells in other animals. For instance, neoblasts have cytoplasmic chromatoid bodies, which are electron-dense structures morphologically similar to germline granules in other animals. Neoblasts express many genes homologous to genes encoding germ-line granule components in other organisms, especially RNA-binding proteins, such as cbc-1 (a Me31B homolog), vasa and tudor. One of those genes, piwi, is predominantly expressed in the neoblasts in planarians. piwi was originally identified as a gene which was required for the maintenance of germ-line stem cells in the ovary and testis in *Drosophila*. Generally, it is thought that Piwi proteins suppress transposable elements to protect the integrity of the genome by acting at the transcriptional level in the nucleus and/or the translational level in the cytoplasm by interacting with short non-coding RNA (piRNA) in sequence-specific manners. Therefore, determination of the sequences of piRNAs would help to identify candidate target genes of Piwi protein. In planarians, piwi genes have important roles in the maintenance and differentiation of the neoblasts. Especially, DjpiwiB(RNAi) animals show serious defects in their regeneration. However, the target genes of DjpiwiB and the molecular mechanism of its function in pluripotency of the neoblasts remain unclear. In this research, we aimed to identify DjPiwiB targets to clarify the molecular function of DjpiwiB in the pluripotency of the neoblasts. Among the four DjPiwi proteins expressed in the neoblasts, only DjPiwiB protein was a nuclear-localized protein, which was observed in almost all cells. In DjpiwiB (RNAi) animals the DjPiwiB protein specifically disappeared from the nuclei of the neoblasts. Although the neoblasts in DjpiwiB (RNAi) animals could proliferate, they lost differentiative ability. These facts indicate that DjPiwiB protein has essential roles in the maintenance and/or exertion of the pluripotency of the neoblasts. To identify the targets of DjPiwiB, we performed immunoprecipitation using anti-DjPiwiB antibody and thereby obtained small (around 32 nt) DjPiwiB-interacting RNAs. Then we sequenced them using an Illumina sequencer and obtained 4,177,817 reads, which showed some typical features of piRNA. We focused on the piRNAs corresponding to our EST database, which were 5% of all piRNAs reads. About 34% of these piRNAs corresponded to transposable elements, for example, Polinton, Gypsy and Penelope, whose expression levels tended to be up-regulated in DjpiwiB (RNAi) animals, suggesting that the main targets of DjPiwiB might be transposable elements, and that DjPiwiB as well as nucleus-localized Piwi proteins in germ-line cells of other animals might suppress transposable elements at the transcriptional level in the nucleus to protect the integrity of the genome. Furthermore, we are now performing transcriptome analysis of DjpiwiB (RNAi) animals to identify additional targets of DjPiwiB which should be strongly suppressed in intact animals. Here, we will mainly discuss on target genes of PiwiB protein, which are predicted to influence the pluripotency of the neoblasts.

Chromatin in Stem Cells

Poster Board Number: T-2188

TRANSCRIPTIONAL CONTROL OF ADULT NEURAL STEM CELL QUIESCENCE

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The capacity to produce neurons throughout life with the demand of stem-cell maintenance is central to normal development and aging. How neural stem cell homeostatic processes in the adult mammalian brain are coordinated is not well understood. Errors in this process can disrupt mature neural circuits and cause defects that affect learning, olfaction and behavior. In our studies aimed at identifying novel regulators of neural stem cell self-renewal and differentiation, we focused on the transcription factor REST (Restrictive Element 1 Silencing Transcription factor), which mediates gene repression by recruiting histone deacetylases, demethylases and methyltransferases to cause epigenetic remodeling of chromatin architecture around the REST target genes. More than 1300 genes are confirmed REST targets; moreover, REST targets are highly enriched in neurons, consistent with the wide scope of REST's influence. In recent work, we demonstrated that: 1) REST is expressed in adult neural stem/progenitors and downregulated in committed neuroblasts, 2) it is required cell-autonomously to prevent precocious neuronal differentiation and maintain the adult neural stem cell pool, and 3) co-binding of REST and its effector proteins control the epigenetic chromatin landscape in adult neural stem cells. To gain mechanistic insight into how REST maintains the neural stem cell pool, we have developed an *in vitro* model of neural stem cell quiescence and found that REST is required to prevent proliferation and transition to the neuronal phenotype. These results suggest that studies of REST in our *in vitro* quiescence model could potential reveal the mechanism controlling adult neural stem cell quiescence and what triggers their transition to proliferative stages. Ongoing studies are aimed at identifying the downstream targets of REST that mediate quiescence and co-regulators of REST and elucidating their roles in quiescence.

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IDENTIFICATION OF USP16, A CHROMATIN REGULATOR ABLE TO ANTAGONIZE BMI-1 AND LIMIT MOUSE AND POSSIBLY HUMAN STEM CELL EXPANSION IN DOWN SYNDROME

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Bmi-1 is one of the most important players in the maintenance of self-renewal of stem cells in many adult tissues, including brain, breast and bone marrow. Bmi1 has also been shown to be important for the maintenance of cancer and cancer stem cells in many tumors. We identified a gene, USP16, expressed at low levels in normal hematopoietic stem cells, but highly expressed in non self-renewing progenitor cells. USP16 is a chromatin regulator, able to remove ubiquitin moieties from histone H2A, antagonizing the effect of the Polycomb complex (that includes Bmi-1). We found that USP16 can antagonize the function of Bmi-1 in the bone marrow

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and in mouse embryonic fribroblasts, by reactivating the expression of downstream genes with tumor-suppressor functions, such as p16INK or p19Arf. Expression of USP16 in mouse hematopoietic stem cells reduces the self-renewal ability of these cells. Interestingly, the human USP16 gene is located on chromosome 21, and it is expressed at abnormal high levels in cells from Down Syndrome (DS) patients, presenting three copies (trisomy) of chromosome 21. In line with our expectations, bone marrow samples from human DS patients show a reduced number of hematopoietic stem cells. Moreover, DS mice models trisomic for USP16 (Ts65Dn) have stem cell defects in both the bone marrow and the breast. Conversely, another DS mouse model disomic for USP16 (Ts1cje) show a normal development of both stem cell compartments. Ts65Dn DS mice have reduced numbers of stem cells with reduced ability to proliferate in vitro and to engraft *in vivo*. The overexpression of USP16 in normal mice tissues mimic this effect. Since DS patients develop considerably less solid tumors than expected, we also hypothesize that USP16 is reducing the self-renewal potential not only of stem cells, but also of cancer stem cells, providing a protection from tumor development. Indeed, preliminary data show that USP16 overerexpression in human and mouse breast cancer models reduces tumor cell growth. Future studies are directed towards understanding the precise role of USP16 in cancer stem cells and its use as a possible therapeutic strategy.

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DISTINCT ROLES OF TET1 AND TET2 IN MOUSE EMBRYONIC STEM CELLS

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The TET proteins (TET1, TET2 and TET3) constitute a new family of dioxygenases that utilize molecular oxygen and the cofactors Fe(II) and 2-oxoglutarate to oxidise 5mC to 5-hydroxymethylcytosine (5hmC) in DNA. The functions of these proteins have been intensively investigated in mouse embryonic stem cells (mESC), which express Tet1 and Tet2 and contain high levels of 5hmC. The 5hmC and mRNA levels of Tet proteins are tightly controlled during mESC differentiation, with decreasing of 5hmC, downregulation of *Tet1* and *Tet2* and upregulation of *Tet3* mRNA respectively. Depletion of Tet1 in mESC skewed differentiation towards the trophectoderm lineage displayed by mESC; moreover Tet1 depletion resulted in decreased expression of the Nodal antagonist Lefty, thereby skewing mESC differentiation toward mesendoderm both in culture and in teratoma assays. However, Tet1 depletion in mES cells resulted in only a partial loss of 5hmC. Tet2 mRNA is expressed at lower levels than Tet1 mRNA in mESC, and Tet2-depleted mESC show a different behavior in teratoma assays compared to Tet1-depleted mESC. We have examined gene expression and 5hmC distribution regulated by Tet1 and Tet2 in mESC, and find that these proteins have distinct roles. Tet2 regulates 5hmC at gene bodies of highly expressed genes, whereasTet1 regulates 5hmC at both gene bodies and TSS. Genes with Tet1-regulated 5hmC at TSS tend to be expressed at lower levels and to be associated with bivalent marks and polycomb repression complex (PRC); whereas genes with Tet2-regulated 5hmC at TSS are expressed at higher levels and show less overlap with bivalent marks and PRC. Moreover, Tet1 and Tet2 play distinct roles in regulating the splicing pathway. Together, these data suggest that Tet1 and Tet2 play different roles in mESC.

Poster Board Number: T-2191

PONTIN IS ESSENTIAL FOR MURINE HEMATOPOIETIC STEM CELL SURVIVAL

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Pontin is a highly conserved DNA helicase/ATPase, which is a component of several macromolecular complexes with functions that include DNA repair, telomere maintenance and tumor suppression. While Pontin is known to be essential in yeast, fruit flies and frogs, its physiological role in mammalian organisms remains to be determined. We here find that Pontin is highly expressed in embryonic stem cells and hematopoietic tissues. Through germline inactivation of Ruvbl1, the gene encoding Pontin, we found it to be essential for early embryogenesis, as Ruvbl1 null embryos could not be recovered beyond the blastocyst stage, where proliferation of the pluripotent inner cell mass was impaired. Conditional ablation of Ruvbl1 in hematopoietic tissues led to bone marrow failure. Competitive repopulation experiments showed that this included the loss of hematopoietic stem cells through apopotosis. Pontin is therefore essential for the function of both embryonic pluripotent cells and adult hematopoietic stem cells.

Poster Board Number: T-2192

EPIGENETIC SWITCH ON THE DIFFERENTIATION OF MOUSE VASCULAR ENDOTHELIAL CELLS

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During the last decades, most studies of vascular development have consisted of gene knockout and knockdown studies using mice and zebrafish. Although these works led to the discoveries of some important factors in vascular development, they could not sufficiently identify the conditions required for vascular formation. To clarify the molecular and epigenetic mechanisms underlying vascular development, we have developed a novel embryonic stem (ES) cell differentiation system. Using this system, we can systematically induce vascular cells *in vitro* and dissect their differentiating processes in detail. 96 hours after induction of differentiation from mouse ES cells, Flk (VEGF, vascular endothelial cell growth factors, receptor 2) -positive mesoderm cells are sorted by MACS using anti-Flk antibody. If these cells are stimulated by VEGF (50ng/ml), cells have commitment to endothelial cells (EC). On the other hand, cells differentiate to smooth muscle cells (SMC) without VEGF stimuli. To elucidate comprehensive gene expression profiles during EC or SMC differentiation, we performed sequential DNA microarray experiments 6,12,24,48 hours after with or without VEGF stimulation. Then we statistically calculated each probe signal value about VEGF (+) / VEGF (-), then selected significantly expression genes at each time point. At a result, transcription factors (Gata2, Etv2, Sox18) were induced at early time points, cell-specific markers (Icam2, VE-Cadherin, Endomucin) were induced at late time points. For example, Etv2 was early induced at 6h VEGF (+), then downregulated, which is consistent of previous reports. Other ets family protein, Fli1 was induced at 12h VEGF (+), Erg was induced at 48h VEGF (+). Our data have suggested that precise mechanisms of temporal and special different ets protein expressions might be necessary for EC differentiation. Next, in order to depict epigenetic landscape

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about vascular development, we conducted ChIP-seq (Chromatin immunoprecipitation with next generation sequencing) using H3K4me3 and H3K27me3 specific antibodies. In general, H3K4me3 marks represent active promoter regions or H3K27me3 marks represent repressive regions and bivalent (both positive H3K4me3 and H3K27me3 marks) marks mean master transcription factors for differentiation. From ChIP-seq, we found out H3K4me3 have strongly correlation with gene expression profiles such as endothelial specific genes. On the other hand, H3K27me3 disappeared after with or without VEGF stimulation. Moreover, we discovered some bivalent genes such as Etv2, Gata2, Sox18 which are upregulated at early time points and supposed to be candidates for master regulators of EC differentiation. These findings have suggested that determination of cell fate is based on not only master transcription factors but also epigenetic histone modifications. Overall, we have demonstrated EC specific epigenetic switch on the differentiation from mouse embryonic stem cells.

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SET NUCLEAR ONCOGENE IS ESSENTIAL FOR MOUSE EMBRYONIC STEM CELL DIFFERENTIATION

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Embryonic stem cells (ESCs) are unique in their ability to self-renew and differentiate into all cell types of the organism. A core set of transcription factors in concert with chromatin regulators maintains the "stem cell state". However this list is not exhaustive and our knowledge about proteins that play important roles in ESCs biology is still limited. To identify novel regulators involved in stem cell maintenance and differentiation, we performed a screen for proteins that are specifically down-regulated during early stages of ES cell differentiation. To this end, we created a library of ESC clones using a gene-tagging approach, with each clone expressing a YFP tagged protein under the control of its own endogenous promoter. Retinoic acid (RA) induced differentiation of gene-tagged clones was used as a model system in combination with time-lapse imaging to measure changes in protein levels that are significantly decreased during differentiation. Using this approach, we identified candidate genes and systematically investigated their role in ESC function. One of the candidates we identified is SET nuclear oncogene (SET), a multifunctional linker histone chaperone. SET has been implicated in a wide array of cellular processes including histone acetylation, DNA replication and cell cycle regulation. Here we show a novel role for SET as a repressor of developmental genes in ESCs. By using various biochemical and genetic methods, we show that SET is essential for active proliferation and differentiation of ESCs. Interestingly, SET function is isoform specific. During differentiation, SET isoform-α levels decline rapidly with concomitant increase in the levels of isoform-β. Hence, SET joins the league of recently identified proteins with alternative functional isoforms in ESCs and differentiated cells. Microarray analysis revealed that expression of important pluripotency markers was not efficiently silenced in SET knockdown cells upon differentiation. We further show that SET is required for proper differentiation of ESCs. Knockdown of SET in ESCs results in upregulation of mesodermal lineage genes, indicating that SET acts as a mesodermal repressor in ESCs. Furthermore, SET depleted ESCs fail to differentiate into neuronal lineage upon induction with RA, suggesting a role in facilitating ectodermal differentiation. Taken together, our data identify a novel chromatin regulator of differentiation in ESCs.

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GLOBAL ANALYSIS OF HIGHER-ORDER CHROMATIN STRUCTURE IN PLURIPOTENT STEM CELLS

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Global analysis of histone modifications and electron microscopic observations have shown that induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs) have a more open (euchromatic) chromatin structure than somatic cells. This fact indicates that the reprogramming process involves dynamic changes of chromatin structures. However, it remains unclear how chromatin conformation affects the pluripotency. Here, we report the characterization of chromatin interactions in pluripotent stem cells. First, we investigated the differences in chromatin interactions among mouse embryonic fibroblasts (MEFs), mouse iPSCs and mouse embryonic stem cells (ESCs) by the chromosome conformation capture (3C), which can detect chromatin loci in close physical proximity. As a result, the interactions between Nanog promoter region and its upstream regions occurred at higher frequencies in mouse ESCs and iPSCs than in MEFs. The different interactions were also observed between human dermal fibroblasts (HDFs) and human iPSCs. These results were consistent with a previous report showing that the chromatin interaction at the Nanog locus in ESCs is dependent on Oct4. Next, we performed the Hi-C, which can determine the genome-wide interactions of chromatins by combining the 3C method with massively parallel sequencing. We identified several different intrachromosomal interactions between MEFs and mouse pluripotent stem cells. In both mouse pluripotent stem cells and MEFs, the probability of the intrachromosomal interaction was higher than that of interchromosomal interactions. In addition, we found that the mouse ESCs and iPSCs had more diverse interchromosomal interactions than MEFs. These findings suggested that the chromatin interactions in somatic cells are dynamically changed during reprogramming process. This study will provide new insights into the molecular basis underlying the iPSCs generation.

Poster Board Number: T-2195

INTEGRATED ANALYSIS OF LNCRNA-CHROMATIN INTERACTION

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Long noncoding RNAs (lncRNAs) are key regulators of chromatin state, yet the nature and sites of RNA-chromatin interaction are mostly unknown. Furthermore, the full cast of lncRNA chromatin regulators remains to be defined. Here we introduce an integrated analysis pipeline that consists of two techniques: Chromatin RNA-IP (ChRIP) and Chromatin Isolation by RNA Purification (ChIRP). While the former technique allows us to systematically discover all lncRNAs stably associated with the chromatin and likely serving regulatory roles, the latter identifies their binding sites on the genome. Using this pipeline, we have found more than 20 stably expressed chromatin-associated lncRNAs in primary human fibroblast, including well-documented examples such as XIST and KCNQ1OT1. The chromatin content is significantly enriched in lncRNAs. In addition, the genomic binding sites of three well-characterized lncRNAs, roX2, TERC and HOTAIR have also been enumerated, which provided critical insights into their mechanisms of action. The ChRIP-

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ChIRP pipeline is a powerful tool that allows us to probe into the dark matter of the genome.

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ROLES OF PHC2 POLYMERIZATION IN POLYCOMB REPRESSIVE FUNCTIONS

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Polycomb group (PcG) proteins mediate heritable but reversible silencing of developmental regulator genes by modifying their chromatin configuration. Accumulating evidence documents a role for PcG proteins in regulating higher order chromatin structures, but the mechanisms and impact of such structures on transcriptional regulation remain obscure. In this study, we identified PcG bodies in mouse primary fibroblasts as distinct foci, at which PRC1 and H3K27me3 are colocalized and canonical PcG target genes are condensed. We found that PcG body formation requires Phc2-SAM polymerization, which critically contributes to condensation and repression of PcG target genes. We further show that Phc2-SAM polymerization limits the dynamic nature of PRC1, and thereby promotes stable association of PRC1 with PcG target genes. Our findings suggest a novel model by which SAM polymerization of Phc2 modulates the structural organization of PcG complexes to enable robust yet reversible PcG-mediated repression during development.

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ROLE OF RING1A/B-MEDIATED HISTONE H2A UBIQUITINATION IN EMBRYONIC AND EPIBLAST STEM CELLS

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Two distinct Polycomb complexes, PRC1 and PRC2, collaborate to maintain epigenetic repression of key developmental loci in embryonic stem cells (ESCs). PRC1 and PRC2 have histone modifying activities, catalyzing mono-ubiquitination of histone H2A (H2AK119u1) and trimethylation of H3 lysine 27 (H3K27me3) respectively. Compared to H3K27me3, localization and role of H2AK119ub1 is not fully understood. Here we present genome-wide H2AK119u1 maps in ESCs and identify a group of genes at which H2AK119u1 is deposited in a Ring1A/B, core PRC1 components, -dependent manner. These genes are a distinctive subset of genes with H3K27me3 enrichment and are the central targets of Polycomb silencing that are required to maintain ESC identity. We further show that the H2A ubiquitination activity of Ring1 is dispensable for its target binding and its activity to compact chromatin at Hox loci, but is indispensable for efficient repression of target genes and thereby ESC maintenance. The Ring1 ubiquitination activity is also indispensable for the maintenance of epiblast stem cells (EpiSCs). These data demonstrate that H2A ubiquitination is an essential step for PRC1-dependent repression of genes that are crucial for the maintenance of ESC and EpiSC identities.

Poster Board Number: T-2198

EXOGENOUS DIRECT DELIVERY OF COACTIVATOR-ASSOCIATED ARGININE METHYLTRANSFERASE 1 (CARM1) PROTEIN USING CELL-PENETRATING PEPTIDE (CPP) TRANSIENTLY ENHANCES THE PLURIPOTENCY-RELATED GENES BY HISTONE MODIFICATION

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Introduction: Human mesenchymal stem cells (hMSCs) have been considered as a useful resource for cell therapies in clinical trials, but they have a major drawback with respect to limited potentials in proliferation and differentiation. For therapeutic applications of hMSCs, it recently suggests that epigenetic modifications, such as DNA methylation and histone modification, can change the property of stem cells or progenitor cells as a brief regulation on the gene expression patterns of the cells. Many researchers introduced the gene transfer method to change effectively the epigenetic modification. But genetic manipulation may cause fatal risks and genetic mutations, which limits clinical applications. In this study, we used bio-friendly protein direct delivery method, which can avoid side effects that come with any genomic manipulation of hMSCs, and transiently alter the regulation on the gene expression patterns. To deliver effectively into hMSCs, we used CPP conjugated to N-terminal of CARM1 protein that is known to up-regulate the pluripotency-related genes by chromatin remodeling through histone arginine methylation. Our results provide an alternative and safe strategy for the changing of epigenetic modification of hMSCs that can be used to facilitate cell therapeutic applications Materials and Methods: Production of the CPP (R7)-CARM1 protein were performed by cloning using pET protein expression vector, and it was purified through 6x His-taq conjugated in C-terminal. This CPP-CARM1 was treated to cultured bone marrow (BM)-, adipose (AD)-hMSCs. The existence of CPP-CARM1 in the nuclei was analyzed by immunocytochemistry using anti-6x His-taq antibody. Also, we performed immunocytochemistry and Western blot analysis using anti-Histone H3 asymmetric dimethyl R17 (H3R17di-me) antibody to confirm functional activity of CPP-CARM1. For genome-wide gene expression profiling, microarray was performed, and up-regulation of the pluripotency related genes were inspected by ChIP. Results: CPP-CARM1 was localized in both cytoplasm and nuclei of hMSCs after 6hr of protein treatment and remained in nuclei only at 12 hr. In groups treated with CPP-CARM1, di-methylation of histone H3R17 were more increased, compared to control group, and the higher levels of methylation were maintained at 12hr and 24hr after treatment. Thus we suggest that the CARM1 protein delivered by the CPP system is biologically active in hMSCs. Genome-wide gene expression profiling supported that delivered CPP-CARM1 protein can cause chromatin remodeling through histone methylation. ChIP assay showed that hMSCs treated with CPP-CARM1 overexpressed the pluripotency related genes promoter, such as OCT4, NANOG, and SOX2. Conclusion: We established a direct delivery system of CARM1 protein using cell-penetrating peptide (R7), and resulted in successful delivery into the nuclei of hMSC. Our results suggest that CPP-CARM1 can alter the global gene expression pattern of hMSCs by histon modification, and especially up-regulate the pluripotency related genes. Therefore this system using CPP is a useful tool for exogenous protein delivery in clinical applications of cell-based therapy.

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GENE REGULATION BY POLYCOMB GROUP PROTEINS IN THE NEOCORTICAL NEURAL PRECURSOR CELLS

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During neocortical development, neural precursor cells (NPCs, or neural stem cells) produce various types of neurons and glial cells in a sequential manner. Although the timing of the fate switch during this sequential process is critical for determining the number of each cell type, the mechanisms are not fully understood. We have previously shown that the polycomb group complex (PcG) restricts neurogenic competence of NPCs and promotes the transition of NPC fate from neurogenic to astrogliogenic. We also found that the level of histone H3K27trimethylation (H3K27me3) increases at the promoter of the proneural gene neurogenin 1 over time during development and that inactivation of PcG by knockout of the Ring1B or Ezh2 gene in the late stage of neocortical development resulted in the increase of ngn1 expression. In the present study, we carried out a genome-wide analysis of H3K27me3 and gene expression in different stages of NPCs during the course of neocortical development. Comparison of H3K27me3 levels and gene expression levels among these different stages of NPCs revealed that PcG regulates a specific subset of neuronal differentiation-related genes. Based on these studies, we will discuss PcG mediated gene regulation during development, in relation to the regulation of RNA polymerase II.

Poster Board Number: T-2200

IDENTIFICATION OF A NOVEL INTRONIC ENHANCER RESPONSIBLE FOR THE TRANSCRIPTIONAL REGULATION OF MUSASHI1 IN NEURAL STEM/PROGENITOR CELLS

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Abstract: The specific genetic regulation of neural primordial cell determination is of great interest in stem cell biology. The Musashi1 (Msi1) protein, which belongs to an evolutionarily conserved family of RNA-binding proteins, is a marker for neural stem/progenitor cells (NS/PCs) in the embryonic and post-natal central nervous system (CNS). Msi1 regulates the translation of its downstream targets, including m-Numb and p21 mRNAs. *In vitro* experiments using knockout mice have shown that Msi1 and its isoform Musashi2 (Msi2) keep NS/PCs in an undifferentiated and proliferative state. Msi1 is expressed not only in NS/PCs, but also in other somatic stem cells and in tumours. However, the mechanisms regulating Msi1 expression are not yet clear. Results: To identify the DNA region affecting Msi1 transcription, we inserted the fusion gene ffLuc, comprised of the fluorescent Venus protein and firefly Luciferase, at the translation initiation site of the mouse Msi1 gene locus contained in a 184-kb bacterial artificial chromosome (BAC). By introducing deletions into the BAC reporter gene and conducting further reporter experiments using a minimized enhancer region determined from H3K4me1 marked site and p300 binding site, we identified a region, "D5E2," that is responsible for Msi1 transcription in NS/PCs. Conclusions: A regulatory element for Msi1 transcription in NS/PCs is located in the sixth intron of the Msi1 gene. The 595-bp D5E2 intronic enhancer can transactivate Msi1 gene expression in CNS with cell-type specificity markedly similar to the endogenous Msi1 expression patterns.

Mesenchymal Cell Lineage Analysis

Poster Board Number: T-2201

IDENTIFICATION OF A PREDICTIVE MOLECULAR SIGNATURE FOR *EX VIVO* MIGRATION OF HUMAN MESENCHYMAL STROMAL CELLS

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Bone marrow-derived mesenchymal stromal cells (MSC) are promising candidate cells in regenerative therapies as they are easily obtained, have the potential to differentiate into several cell types, and show immunomodulatory properties. However, previous studies have demonstrated that human MSC exhibit poor homing capacity to bone following i.v. infusion, possibly due to the heterogeneity within a MSC population, constituting cells with different ability for homing. We have recently studied the cellular heterogeneity of MSC in relation to bone forming ability by isolating several single MSC cell clones based on their ectopic *in vivo* high- (HBF) and low bone-forming (LBF) capacity. To investigate whether MSC clones would also maintain a predictive molecular signature for better migration and engraftment *in vivo*, we performed Illumina®-based microarray analysis comparing three HBF versus three LBF clones. Among 746 differentially up-regulated genes (p value < 0.01, 1.5-fold cut off), around 14 percent of genes were annotated as potentially involved in stem cell homing (chemoattraction, adhesion, migration), including chemokine (C-C motif) ligand 8 (CCL8), CCL13, chemokine (C-X-C motif) ligand 16 (CXCL16), CXC receptor 7, insulin-like growth factor receptor (IGFR), alpha 1 integrin, interleukin 8, vascular cell adhesion molecule 1, stromal cell-derived factor 1 (SDF1), platelet-derived growth factor receptor (PDGFR) A and B, and several members of the tumor necrosis factor super family. In consistence with this molecular signature, HBF-clones showed significant in vitro transwell migration toward SDF1, IGF1, and PDGFbb as compared to LBF-clones (116.2±12.1% vs 101.2±1.9%; 124.9±8.6% vs 106.4±3.5%; 160.8±8.1 vs 139.0±5.2%, respectively). In conclusion, our data demonstrated the existence of cellular heterogeneity in relation to the migration ability of human bone marrow derived MSC and a positive correlation between responsiveness towards chemoattractants and bone forming capacity. Furthermore, we identified a predictive molecular phenotype that may enable isolation of MSC sub-populations with high bone homing capacity suitable for bone regeneration.

Poster Board Number: T-2202

THE HUMAN MESENCHYMAL STEM CELLS DERIVED INDUCED PLURIPOTENT STEM CELLS MAY BE A PROMISING CELL SOURCE FOR ALLOGENEIC IPSCS TRANSPLANTATION THERAPY

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Background: Placental tissue is readily available, easily procured without invasive procedures, and does not elicit ethical debate.

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Human amniotic mesenchymal stem cells (hAMSCs) are isolated from amniotic membrane underlying the chorion of placental. The hAMSCs demonstrated immunomodulatory properties known to suppress host immune responses. As reported in several studies, hAMSCs' immunosuppressive effects were confirmed with positive findings for CD59 and HLA-G. Soluble HLA-G molecules produced by the placenta induced apoptosis of activated CD8+ T-cells and inhibited CD4+ T-cell proliferation. CD59, a complement regulatory protein, prevented complement-mediated cell damage through inhibition of the complement membrane attack complex. Immunologic reaction is a critical issue in stem cell-based therapy when using non-matched stem cell therapy. To address this issue, the immune characteristics of hAMSCs and iPSCs derived from hAMSCs (MiPSCs) were investigated *in vivo* and in vitro in this study. Method and Results: hAMSCs were isolated from human placentas obtained from healthy subjects at the Stanford University Medical Center, Stanford, CA. This population of hAMSCs demonstrated high expression of HLA-G and CD59 by immunohistology and RT-PCR assays. A single polycistronic lentivirus was introduced into hAMSCs to generate MiPSCs. The MiPSCs also expressed high level of HLA-G and CD59 by immunohistology and RT-PCR assays. The immunological property characterized by CD59 +, HLA-G + may suggest that the MiPSCs retain the immunosuppressive properties of the hAMSCs. In in vitro study, the leukocyte-mediated cytotoxicity experiments revealed that MiPSCs and hAMSCs both inhibited the immune rejection of leukocytes in vitro. To investigate the post-transplantation survival *in vivo*, the mESCs, hAMSCs and MiPSCs with luciferase reporter gene were injected into hind limbs of immunocompetent SVJ mice. The *in vivo* study demonstrated robust BLI survival signal by the luciferase-transduced mESCs and hAMSCs at week 1, whereas MiPSCs didn't survive in the SVJ mouse. Conclusions: The generation of induced pluripotent cells (iPSCs) from differentiated adult cells has vast therapeutic implications in regenerative medicine, then which is hampered by immune rejection of post-transplantation. Our data revealed that hAMSCs were able to be survival in immunocompetent SVJ mice, which suggested HLA-G and CD59 might play an important role in immunosuppressive effects. Although the hAMSCs derived MiPSCs didn't show the survival signals *in vivo* as well as hAMSCs, MiPSCs retained the unique immune properties of hAMSCs and inhibited immune rejection in vitro that both have been verified in our study. The reasons of poor survival of MiPSCs are not clear, but one of which may be due to changes of immune profiles of MiPSCs in differentiation process. This represents a major challenge for iPSCs transplantation therapy, which may be addressed by the immunosuppressive effects of HLA-G and CD59. In our future works, the MiPSCs' immune properties will be modified by enhancing expression of HLA-G and CD59 to improve survival of MiPSCs in immunocompetent SVJ mice. The study could make allogeneic iPSCs transplantation therapy possible.

Poster Board Number: T-2203

EVALUATING THE DIFFERENTIATION POTENTIAL OF HUMAN SOMATIC CELLS IN MOUSE FOETUSES.

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We employed a two-phased *ex vivo* culture protocol in which cells from adult human olfactory mucosal biopsies were enriched to apparent homogeneity. The resulting cells exhibited a bipolar, mesenchymal morphology and were amenable to stable genetic modification by electroporation with plasmid DNA. Transcriptome analyses of Phase 1- and Phase 2-cultured cells revealed that signalling through the interferon pathway was active in Phase 1 cells, but was silenced in Phase 2 cells, and the expression of chromatin-modifying genes was induced concomitantly with repressors of transcription in Phase 2 cells, whereas the opposite was true in Phase 1 cells. 19 of 175 pluripotency network gene transcripts were significantly more abundant in Phase 2 cells, including CD44, a marker of mesenchymal stem cells. In contrast, CDK7 and SNRPN were preferentially expressed in Phase 1 cells. The karyotypes of Phase 2 cells of both genetic sexes were normal, suggesting that the two-stage culture conditions did not cause detectable chromosomal abnormalities.The plasticity of cultured adult human olfactory cells *in vivo,* was tested by blastocyst complementation. C57BL/6J host foetuses were recovered at 16.5 days post coitus (dpc), the designated experimental endpoint. At this stage, the tissues were functionally immature; however body morphogenesis and tissue architecture were well established. Immunochemical analysis confirmed that cultured adult human olfactory cells contributed to the developing midbrain and structures elsewhere within the CNS, myocardium, skin, eye primordium, skeletal muscle, connective tissue, adipose tissue, chondrocytes, lung, chorionic villi, placenta and the developing testis. Adult human olfactory cells were not detected in the liver of foetuses that were examined. Thus, the foetal mouse would appear to be a useful model in which to evaluate human somatic cell lineage commitment and early differentiation *in vivo*.

Poster Board Number: T-2204

EFFECTS OF OXIDATIVE STRESS ON HUMAN MESENCHYMAL STEM CELL PREMATURE SENESCENCE

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Aim: Mesenchymal stem cells (MSCs) often arrive at cellular senescence during in vitro expansion by experimental multiple stimuli including oxidative stress. In this study, we investigated protein markers associated with cellular senescence upon premature senescence of MSCs due to oxidative stress and the effect of resveratrol (RSV) against their premature senescence. Methods: After MSCs (Lonza) were exposed to 200 μM H2O2, the expression or activation levels of SIRT1, p21, pERK1/2 and ppRb were measured using real-time RT-PCR and western blot. Further, after RSV (5 and 25 μM) was pretreated in MSCs before 200 μM H2O2 exposure, the expression or activation levels of SIRT1, p21, pERK1/2 and ppRb were measured. Results: The proliferation of MSCs in response to H2O2 was

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decreased, while senescence-associated β-galactosidase (SA-β-gal) activity of the cells was increased, indicating that exposure of MSCs to H2O2 induced premature senescence of the cells. Premature senescence of MSCs in response to H2O2 induced the decrease of SIRT1activity (indirectly identified by measuring acetylated Lys-9 of histone H3) as well as the decrease of SIRT1expression. p21 and pERK1/2 in the cells in response to H2O2 were increased, while ppRb was decreased in a dose-dependent manner. On the other hand, RSV pretreatment decreased premature senescence of MSCs. In addition, RSV pretreatment before exposing the cells to H2O2 alleviated the alteration of the proteins (SIRT1, p21 and pERK1/2) sensitive to H2O2 treatment. Conclusion: Our results suggest that oxidative stress including H2O2 exposure induces not only premature senescence of MSCs in vitro but also alteration of senescence-associated proteins. Further, MSCs may exhibit an increased tolerance against oxidative stress via senescence-associated proteins regulated by RSV.

Poster Board Number: T-2205

MICRORNA PROFILE ANALYSIS OF HUMAN MESENCHYMAL STEM CELLS FROM DIVERSE ORIGINS

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Most miRNAs are evolutionarily conserved in related species and many miRNAs have well-defined developmental and cell-type-specific expression patterns. Mesencymal stem cells (MSCs) have been isolated from various human tissues and been used as powerful resources for further clinical therapies. However, the differences between MSCs-derirved from different sources are still understudied. Here, we show the miRNA expression profiles and analyze the diverse pattern of MSCs derived from bone marrow (BM), umbilical cord blood (UCB), adipose tissue (AT), amniotic membrane (AM) and amniotic fluid (AF). Our results indicate that within each group of MSCs from the same origin, the variability of the miRNA expression levels is smaller than that between groups of different origins. 83 of 887 miRNAs show various expressions between these five MSCs groups. Clustering the 83 miRNAs, the correlation between different origins reveals that MSCs-derived from umbilical cord blood and from bone marrow are more homogenous than other sources. The phylogenetic tree based on the similarity indicates two crucial stages in MSCs miRNA evolution. The first divergence is AF miRNAs from other sources, and the hemotopoietic origin separates BM miRNAs and UCB miRNAs from AM miRNAs and AT miRNAs. The further analysis for the differences of miRNA expression between hemotopoietic lineages and other origins filter 11 miRNAs targets out. 7 of 11 miRNAs can be predicted to involve in hematopoietic cell differentiated pathway, and the top four miRNAs (hsa-miR-424, has-miR-34c-5p, has-miR-503 and has-miR-1274a) target individually 7 to 10 genes for regulating hematopoietic differentiation in silico. In this study, we suggest that the different miRNA expression profiles can be analyzed from different MSCs origins and the AF is the most diverse source than others. The diversity of miRNA expression can be used in systematic analysis for the origin specific regulation researches.

Poster Board Number: T-2206

GENE EXPRESSION ANALYSIS OF HUMAN MULTIPOTENT MESENCHYMAL STROMAL CELLS DERIVED FROM BONE MARROW OF OSTEOGENESIS IMPERFECTA PATIENTS DURING OSTEOBLAST DIFFERENTIATION.

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Osteogenesis imperfecta (OI) is characterized as a genetic disorder in which a generalized osteopenia leads to short stature, bone fragility and serious skeletal deformities. Mesenchymal stem cells (MSCs) are precursors present in adult bone marrow that can differentiate into osteoblasts, adipocytes and myoblasts that have been given great importance as a source cell therapy. The aim of this study was to analyze the gene expression profile during osteogenic differentiation from mesenchymal stem cells from bone marrow taken from patients diagnosed with Osteogenesis Imperfecta and control subjects. Samples were collected from three normal individuals and five samples from patients with Osteogenesis Imperfecta. Mononuclear cells (MON) were isolated to obtain mesenchymal cells that were expanded until third passage when the stimulus for osteogenic differentiation was induced. Analyses were also conducted to count the CFU-F and for four of the five samples from patients with OI, the number of CFU-F observed was lower than generally found for normal samples. Cells were collected for analysis of cell immunophenotyping by flow cytometry and RNA was extracted from the resulting sample called T0. Remaining cells were stimulated for osteogenic differentiation. After a day in culture with stimulation, cells from another bottle had their RNA extracted (T1), and the same procedure was performed on days 2 (T2), 7 (T7), 12 (T12), 17 (T17) and 21 (T21). All samples have shown potential of *in vitro* differentiation into osteoblasts and adipocytes. Immunophenotyping of mesenchymal cells was performed and samples of all patients had immunophenotypic profile consistent with previous works. We identified mutations in *COL1A1* and / or *COL1A2* responsible for developing the disease for four of five patients. For the patient with Osteogenesis Imperfecta and Bruck Syndrome, coding region of the gene *PLOD2* was also sequenced, but no mutations were found. The gene expression analysis was performed by microarray and identified several genes with differential expression. Some genes of fundamental importance in osteoblast differentiation showed lower expression in samples from patients with OI, suggesting a minor involvement of MSCs of patients with osteogenic lineage. Other genes also confirmed their differential expression by Real Time PCR. We observed an increased expression of genes related to adipocytes, suggesting an increased adipogenic differentiation at the expense of osteogenic differentiation. The expression of *PLOD2* gene variants proved to be different between normal samples, OI and the patient with Bruck Syndrome. There was also evidence of differential expression of 29b microRNA, with established role during osteogenic differentiation, suggesting a mechanism dependent regulation of mRNA abundance of its gene target, *COL1A1*.

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Poster Board Number: T-2207

DNA METHYLATION PROFILING OF HUMAN BM-MSCS IN LONG-TERM CULTURE

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Human bone marrow mesenchymal stem cells (MSCs) expanded *in vitro* exhibit not only a tendency to lose their proliferative potential, homing ability and telomere length but also genetic or epigenetic modifications, resulting in senescence. We compared differential methylation patterns of genes and miRNAs between early-passage (passage 5 (P5)) and late-passage (passage 15 (P15)) cells and estimated the relationship between senescence and DNA methylation patterns. When we examined hypermethylated genes (methylation peak ≥ 2) at P5 or P15, 2,739 genes, including those related to fructose and mannose metabolism and calcium signaling pathways, and 2,587 genes, including those related to DNA replication, cell cycle and the PPAR signaling pathway, were hypermethylated at P5 and P15, respectively. There was common hypermethylation of 1,205 genes at both P5 and P15. In addition, genes that were hypermethylated at P5 (CPEB1, GMPPA, CDKN1A, TBX2, SMAD9 and MCM2) showed lower mRNA expression than did those hypermethylated at P15, whereas genes that were hypermethylated at P15 (MAML2, FEN1 and CDK4) showed lower mRNA expression than did those that were hypermethylated at P5, demonstrating that hypermethylation at DNA promoter regions inhibited gene expression and that hypomethylation increased gene expression. In the case of hypermethylation on miRNA, 27 miRNAs were hypermethylated at P5, whereas 44 miRNAs were hypermethylated at P15. These results show that hypermethylation increases at genes related to DNA replication, cell cycle and adipogenic differentiation due to long-term culture, which may in part affect MSC senescence.

Poster Board Number: T-2208

HUMAN BLOOD-DERIVED MESENCHYMAL STEM CELLS ARE ASSEMBLED

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We have found that a group of RNA-containing particles in human umbilical cord blood and mouse blood. These particles contain RNA, but not DNA, fragmented less than 200 nt and; more than 30% of these RNAs are micro-RNA. After relocate into lipid membrane vesicles, these particles grow from less than 2 μm up to 10 μm and become living organisms, which we termed Aidars. Video records show that mature Aidars have a flexible membrane and amoebic-like movement. They increase their size by taking materials through a process similar to endocytosis and phagocytosis. Mature Aidars also take materials from the nuclear areas of the adjacent eukaryotic cells, however, without harming these cells. Further, mature Aidars fuse together to increase their body sizes and become eukaryotic cells. FACS analysis and immunofluorescent studies demonstrate that most Aidars express integrin ß1, sox-2 and VASA. Electron microscopy and stains also confirm that Aidars do not have nucleus. Our data provide the evidence that circulation RNA particles together with cellular membrane vesicles and proteins can self-assemble into a group of living organisms. These organisms fuse and, after taken the genetic materials of the eukaryotic cell, transform into blood-derived mesenchymal stem cells.

Poster Board Number: T-2209

A MODIFIED METHOD FOR ISOLATION AND PROPAGATION OF MOUSE BONE MARROW DERIVED-MESENCHYMAL STEM CELLS

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Bone marrow derived-mesenchymal stem cells (MSCs) also, called mesenchymal stromal cells have captured substantial scientific and public interest because of their suitability in the context of physical and immune injuries, to regenerate tissues either by producing bioactive molecules such as growth- and differentiation factors or by differentiating themselves into damage-specific cells/tissiues. MSCs have been cultured in different media supplemented with various cytokines and growth factors as well as on various surfaces modified with extracellular components like collagen, fibronectin, poly-L- lysine etc., aiming to improve and understand their biology of self-renewal, proliferation and differentiation. In similar direction, we present a novel method for culture of mesenchymal stem cells derived from the mouse bone marrow on an extracellular matrix-independent, scratch-based modified surface in Dulbecco's modified Eagle's medium with high glucose (DMEM-HG) and 15 % fetal bovine serum (FBS) in a 37^0 C, 5% CO_2 incubator. On comparison with the cells grown on the unmodified surface, we observed three to four fold increase in the cell yield. Apart from the surface-modification, we also observed trypsinization and cell harvesting to be very easy by using a pre-warm trypsin and phosphate buffered saline (PBS) solution. A higher number of the pure population of spindle-shaped cells expressed the MSC-specific markers like CD29, CD44, Sca-1 and were devoid of hematopoietic cells as they were negative for the markers, CD34, CD45 and CD11b. The MSCs also demonstrated full potential for differentiation into mesodermal cells like adipocytes, osteocytes and chondrocytes in culture. This simple method can be used for isolation, culture and propagation of MSCs from the bone marrow.

Poster Board Number: T-2210

GROWTH AND PHENOTYPIC CHARACTERISTIC OF MURINE NAIL MATRIX CELL CULTURE

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Objective. The search for new sources of stem cells that can be used in regenerative medicine is the topic issue for today. Nail matrix probably contains cells with high regenerative potential, because growth and regeneration of nails occurs throughout life. It is known, that the nail matrix can stimulate regeneration of the phalanges of mammals. Moreover, allogenic cells from blood or bone marrow transplants can contribute to formation of humans' nails . But stem cell cultures and localization of these cells in nails are still not enough characterized. Material and methods. Nail matrixes of mice were isolated by microdissection technique under aseptic conditions. Cells were obtained using 0,1% collagenase and plated into dish coated with BDMatrigel. Cells were cultured in DMEM:F12 supplemented with 15% fetal calf serum (FCS), refreshing the medium every 4th day. Subcultivation was performed when monolayer reached approximately 80% confluence. After passage 3 the culture medium was changed to neurobasal medium with 10% FCS and 0,3 ng/ml β-FGF. Phenotyping of the cells was performed using BD FACSAria cell sorter. Results. Cells underwent 6 passages

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and survived *in vitro* for up to 12 weeks. First adhered cells were observed on the day 12 after plating. Primary cell culture consisted of different cell types. There were fibroblast-like cells which did not form dense monolayer and were oriented along longer axes. Polygonal cells formed on the surface many cobblestone-like plaques with typical epithelial morphology. Spindle shaped cells formed net like structures contacting with each other with their long sprouts. Number of these cells increased after passaging. After passage 3 in some cells lipid granules (Oil Red staining) were founded which indicates their spontaneous adipogenic differentiation. Phenotyping of these cells at the 2nd passage showed high level expression of CD44 and CD73 (about 90% both), whereas expression of CD90 was about 45%, and expression of haematopoietic markers was performed at low levels: CD34 - less than 25%, CD 117 - about 13% and CD45 - only 6%. On passage 3 expression of CD44 (100%), CD73 (98%) and CD90 (nearly 90%) increased while expression of CD34, CD 117 and CD45 was less than 2%. Conclusion. We showed that nail matrix cell culture is rather heterogenous and consists of cells with different morphology. Phenotypic characteristic of this culture represented high expression of stromal stem cell markers and low level of haematopoietic markers. We suggest that the nail matrix can be a transient niche for different types of stem cells and can be considered as an alternative repository of adult cells with regenerative potential.

Poster Board Number: T-2211

EFFECTS OF LONG-TERM SEQUENTIAL PASSAGE ON CHARACTERISTICS OF CANINE ADIPOSE DERIVED MESENCHYMAL STEM CELLS

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Adipose-derived mesenchymal stem cells (AD-MSCs) have demonstrated the feasibility in therapeutic application, due to ease of isolation and abundance in the tissue without age barrier with the great potential of differentiation into multi-lineage cells. However, the promising potential is not predictable and may be lost during proliferation of the cells in a large number and sequential passage in a long-term. Therefore, this study was conducted to investigate effect of sequential passage on characteristics of canine AD-MSCs In this study, canine mesenchymal stem cells (cAD-MSCs) were isolated from adipose tissues (n=5) and subjected to 9 sequential passages. The cells at each passage were characterized for properties associated with multipotent MSCs such as proliferation kinetics, expression of MSCs-specific surface markers, expression of molecules associated with self-renewal and differentiation capabilities into mesodermal lineage cells. In results, the proliferation of the cells were peaked at passage 1 and stopped after passage 7, which was evaluated by CPDL, while doubling time was increased significantly after passage 6. Expression of MSCs surface markers (CD44, CD90, and CD105) and molecule (Oct-4, Sox-2, Nanog and HMGA2) associated with self-renewal was decreased significantly after P6. Regarding differentiation capabilities, cells at passage 1 to 9 could be stimulated to undergo adipogenic and chondrogenic differentiation under specific culture conditions. However, the level of adipogenic and chondrogenic differentiation was negatively correlated with the number of subpassage. Conclusively, the present study suggested that sequential passages affect multipotent properties of cAD-MSCs, which should be considered in therapeutic trial of cAD-MSCs.

Poster Board Number: T-2212

CHARACTERIZATION OF COMMON MARMOSET BONE MARROW DERIVED STEM CELLS

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Mesenchymal stem cells and hematopoietic cells, which are present in bone marrow, are expected to be applied to regeneration medicine. In this study, we harvested bone marrow-derived cells from the femur of newborn common marmoset (Callithrix jacchus) males and investigated the characterization of the cells with an aim to develop the research field of regenerative medicine. The cells were cultured in DMEM containing 10% FBS, and cell morphology was observed to resemble that of fibroblasts. Immunofluorescence and western blotting revealed that the cells expressed the mesenchymal markers CD73 and CD90. In addition, *in vitro* differentiation analysis showed that the cells had the potency to differentiate into adipocytes and osteocytes. These results indicate that the cells had characteristics of mesenchymal stem cells. In the future, we will investigate whether the cells retain the characteristics of mesenchymal stem cells with increasing passage number.

Poster Board Number: T-2213

MSCS-INDUCED IMMUNOSUPPRESSION OF TH17 CELLS IS CELL-TO-CELL CONTACT MEDIATED AND DOSE DEPENDENT

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Mesenchymal stem cells (MSCs) are multipotent stem cells with immune-modulatory properties. MSCs produce soluble factors such as IL-6, IL10, TGF-b1 and PGE2 that are involved in their suppressive abilities. In addition, IL-6 and TGF-β1 are also involved in the development of the proinflammatory T helper 17 (Th17) cells. The aim of this study is to determine the impact of MSCs on the proliferation and differentiation of Th17 cells in a co-culture system, with particular interest on unraveling the mechanism behind it. MSCs isolated from mice bone marrow were characterized according to their surface antigens expression and by their multilineage differentiation potential. Mouse CD4+T cells were differentiated into Th17 cells and co-cultured with MSCs at different ratios and in the presence or absence of a transwell culture system. At 6 day post-coculture, the expression level of IL-17 by Th17 cells was measured by flow cytometry. We first show that MSCs exert a dose-dependent suppressive and proinflammatory effect on Th17 cells depending on MSCs/ CD4+T ratio, where a significant inhibition was only observed at the ratio of 1:10 and not in the case of 1:100. The suppression of the differentiation of Th17 cells was cell-to-cell contact mediated as in the presence of a transwell culture system prevented the observed inhibition effect on Th17 . All this data demonstrate, that MSCs exert an immunosuppressive effect on TH17 differentiation , however, the molecules involved in this mechanism are still under investigation and needs to be elucidated to be able to use their properties for the treatment of autoimmune diseases.

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Poster Board Number: T-2214

THE ANCESTRAL ORIGIN OF MESENCHYMAL STEM CELLS IS REVEALED BY PERIVASCULAR SDF1 EXPRESSION

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Mesenchymal stem cells (MSC) are being used in clinical trials to provide immune suppression or assist in tissue repair. Although MSC are mesodermal in origin and function as a "niche cell" in the bone marrow, their definitive origin remains elusive. Stromal derived factor-1 (SDF1), a chemokine critical for hematopoietic stem cell homing, was first discovered in cultured bone marrow stromal cells, presumably MSC. To identify an *in vivo* ancestor to MSC, we utilized the zebrafish model to create an SDF-1 transgenic animal. A 4 kb promoter region of sdf1a upstream of DsRed was cloned to create transgenic zebrafish harboring sdf1a expressing cells identified by DsRed fluorescence. Not unexpectedly, a wide range of tissues expressed SDF1. Prominent perivascular cells expressed DsRed. DsRed positive cells were flow sorted and a culture system was established for in vitro propagation. DsRed2 positive cells expressed elevated levels of transgelin, smooth muscle actin and cadherin 5 compared to flow sorted endothelial cells by qRT-PCR; results consistent with a perivascular cell origin. Cultured perivascular cells showed a fibroblastoid morphology and a doubling time of ~48 hours. Cultured cells were positive for CD90, CD73, CD105 and negative for CD45 by RT-PCR, characteristic for MSC. Cultured perivascular cells were differentiated into the osteoblastic, chondrocytic, and adipocytic lineages over 4 weeks. Cell histochemical staining patterns and multi-gene expression appropriate for each lineage by RT-PCR was observed (exampled by osteocalcin, leptina, and collagen10a1 respectively). Cultured perivascular cells expressed fgfr and responded to bFGF in a dose dependent manner. Genes important hematopoietic cell maintenance including Notch-Delta family members, angiopoietin, and kit-ligand also were expressed. Cultured cells supported zebrafish hematopoietic cells in co-culture conditions and in vitro co-cultured cells engrafted into myeloablated recipient fish. Microarray expression analysis as was performed since such data are not available for perivascular cells of any type. Genes associated with vascular differentiation and vascular repair were found to be expressed. Tandem mass-spectrometry-based proteomic studies on the cultured perivascular cells revealed proteins also found to be expressed in human MSC. In conclusion, we have isolated cells with many of the characteristics of MSC whose origin was perivascular in the correct anatomical location in zebrafish. Such bona fide perivascular MSC will be useful in high throughput experiments. Thus, the sdf1:DsRed zebrafish will be useful tool to study both the role of perivascular cells in vascular function as well as their interaction with hematopoietic cells in future work taking advantage of the power of the zebrafish especially, that of *in vivo* microscopic visualization of cell trafficking.

Poster Board Number: T-2215

BETA-CATENIN IS ESSENTIAL FOR REGENERATIVE ROLE OF MYELOID LINEAGE CELLS DURING WOUND REPAIR.

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Three percent of the population suffers from disordered cutaneous wound repair. This causes morbidity from loss of function, negative psychosocial effects from disfigurement, or even mortality from the loss of the skin's barrier function. During the proliferative phase of wound repair, β-catenin mediated Tcf-dependent transcription is active in a subset of dermal cells. β-catenin mediates the effects of growth factors and regulates the number of fibroblast like cells that accumulate during healing, controlling scar size. Cells in which β-catenin is activated potentially derive from several sources, but the source of these cells and their functional contribution is unknown. Here we showed that Tcf transcriptionally active cells express genes characteristic of myeloid lineage cells during skin healing. Lineage tracing studies showed that myeloid lineage cells contribute 18% of dermal cells during the proliferative phase of murine wound repair. A subpopulation of these cells showed characteristics of mesenchymal cells only in the presence of β-Catenin. Thus, β-Catenin is necessary for myeloid lineage cells to attain characteristics of mesenchymal cells. Mice whose macrophages lack β-Catenin show a significant impairment in wound healing. Injection of wild-type macrophages, but not macrophages lacking β-Catenin, improves deficient wound repair in irradiated skin. Taken together, our results show β-Catenin plays an essential role in myeloid lineage cells during wound repair, suggest a novel cell source and targetable mechanism which can be utilized to improve disordered wound healing.

Poster Board Number: T-2216

REPROGRAMMING TO A PLURIPOTENT STATE MODIFIES MENSTRUAL BLOOD-DERIVED MESENCHYMAL STEM CELL RESISTANCE TO OXIDATIVE STRESS

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Aim: Recently, mesenchymal stem cells (MSCs) were obtained from menstrual blood and proved to be useful to treat diseases in which tissue damage is linked to oxidative stress (OS). Reactive oxygen species (ROS) play a key role in the regulation of cell adhesion, migration, proliferation and it has been demonstrated that ROS inhibit cellular adhesion of transplanted stem cells. Thus, the aim of this work was to evaluate whether human menstrual blood-derived mesenchymal cells (MBMC) are resistant to OS and compare to human embryonic stem cells (H9) and induced pluripotent stem cells derived from MBMC (iPS-MBMC). Methods and Results: Human menstrual blood was collected from twelve healthy female subjects when menstrual flow initiated. All experiments below were approved by our local institutional review board (HUCFF, UFRJ, RJ, Brazil) protocol no: 056/09. MBMC described in this work

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were obtained based on the criteria defined by The International Society for Cellular Therapy position statement. Besides this, MBMC expressed the core embryonic stem (ES) cell regulators Oct4, Sox2, nanog and Klf4. MBMC and human dermal fibroblasts were cultured with increasing H_2O_2 concentrations ranging from 0 to 10000 µM and cell viability was evaluated by MTT assay 24 hours later. The dose (IC_{50}) which kills 50% of the cells was 1812 µM for MBMC and 816 µM for fibroblasts, showing that MBMC are extremely resistant to OS-induced death. Moreover, MBMC produce three times more extracellular H_2O_2 than iPS-MBMC and H9 (5.76; 2.35; 2.09 nmol H_2O_2/h/10^5 cells, respectively), which were detected by Amplex red/HRP assay. This extracellular production of H_2O_2 by MBMC was inhibited by diphenyliodonium (DPI), in a non dose-dependent way, indicating that NADPH oxidases are responsible for this production. To measure intracellular ROS production, cells were incubated with 10 µM of CM-H2DCFDA and fluorescence was measured by flow cytometry. No differences were found in the amount of ROS produced in the cytoplasm of MBMC, iPS-MBMC and H9 in baseline conditions. However, when exogenous H_2O_2 was added, iPS-MBMC and H9 had a significant increase in the cytoplasmic amount of ROS, which did not occur in MBMC. In addition, expression of genes coding for antioxidant enzymes was assessed by real-time RT-PCR. Catalase, SOD2, SOD3 expression was five fold higher in MBMC than in iPS-MBMC and H9, and SOD1 was two fold higher in MBMC than in the other cells. However, GPx1 was only 50% lower in iPS-MBMC when compared to MBMC. Curiously, GPx 3 was approximately 25% higher in H9 cells. In contrast, GPx activity was similar in MBMC, iPS-MBMC and ES cells. Conclusion: MBMC are mesenchymal stem cells and express pluripotency markers, suggesting that they are a powerful source of progenitors and could potentially be differentiated in different mesodermal tissue types. Moreover, MBMC presented high resistance to OS, probably because they are capable of producing ROS, leading to a greater expression of antioxidant enzymes. In contrast, after reprogramming to pluripotent stage, iPS generated from MBMC were not OS resistant and were similar to embryonic stem cells. Therefore, MBMC might be considered a source for cell therapy strategy aimed to treat patients with diseases in which onset and progression is associated with OS.

Poster Board Number: T-2218

GENE TRANSFECTION TO MSCS BY CHITOSAN NANOPARTICLES

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Purpose: This study describes a nonviral gene delivery by low molecular weight chitosan nanoparticles as a novel way of transferring exogenous gene into human marrow-derived mesenchymal stem cells. In this context there is a little information. Methods: The chitosan/DNA nanoparticles were synthesized through the complex coacervation method of the chitosan solution with pTracer-CMV2 plasmid containing fluorescent GFP. In this regard several concentrations of chitosan solution including 1%-0.5%-0.1%-0.05%-0.01%-0.005%-0.001% were used. To examine the synthesis of complexes of nanoparticles, samples were run through an agarose gel. Particle size and their zeta potential were measured by zetasizer. MSCs were prepared from human bone marrow and trasfected with chitosan/plasmid nano particle complex synthesized using above-mentioned dilutions of chitosan. Transfection with Lipofectamine 2000 was taken as the control. The viability of cells was determined by MTT assay and the transfection efficiency was determined by observing the culture under fluorescent microscope. Results: According to our findings 1%-0.5%-0.1%-0.05% concentrations could form chitosan/ pTracer-CMV2 nanoparticle. The particle size was determined as 629, 404, 242 and 416nm respectively. MTT assay was indicated that the average viability of cells treated with chitosan/plasmid nanoparticles was about 96% versus 75% for Lipofectamine 2000. Furthermore the best transfection rate (2%) was achieved in 0.1% chitosan/DNA nanoparticles complex having the size of 243nm. This value was about 4.7% for Lipofectamine 2000. Conclusion: Gene delivery using chitosan nanoparticles is a promising strategy for nonviral gene delivery. Although transfection efficiency of nanoparticle method tended to be lower than that of Lipofectamine 2000 but it appeared safer owing to lower cytotoxic effects on the cell culture. Improving transfection rate of nanoparticle method however needs further investigation.

Poster Board Number: T-2219

SOX2-POSITIVE AND -NEGATIVE DERMAL PAPILLA CELLS MAINTAIN THEIR INTRINSIC DIFFERENCES, INDEPENDENT OF THE MICROENVIRONMENT

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In neonatal mouse skin two types of dermal papilla (DP) can be distinguished on the basis of Sox2 expression. We describe a three-dimensional hydrogel culture system that supports clonal growth of neonatal dermal cells. Disaggreated cell populations formed spheres that expressed DP markers alkaline phosphatase, alpha-8-integrin and CD133. Nevertheless, spheres formed by CD133- cells could not support hair follicle formation in skin reconstitution assays. Sox2+CD133+ and Sox2-CD133+ spheres contributed to the DP of both GAA and ZZ hairs. There was no correlation between sphere size and hair type. Sox2 expression was maintained in culture, but was not induced in Sox2- cells in vitro or *in vivo*, suggesting that Sox2+ cells are a distinct cellular lineage. Although Sox2+ cells were least efficient at forming spheres they had the greatest ability to contribute to DP and non-DP dermis in reconstituted skin. Since the culture system supports clonal growth of DP cells and maintenance of distinct DP cell types, it will be useful for further analysis of the intrinsic and extrinsic signals that control DP function.

Poster Board Number: T-2220

ISOLATION AND CHARACTERIZATION OF MESENCHYMAL STEM CELL FROM REUSABLE AND DISPOSABLE BONE MARROW COLLECTION FILTER AN ETHICAL SOURCE OF CELLS.

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Have been described the presence of at least 2 types of stem cells deriving from bone marrow; a population termed hematopoietic stem and a second population entitled stromal stem cell. Stromal stem cells also known as mesenchymal stem cells (MSC), were firstly described more than 30 years ago, and there has been an increasing interest on studying this cells, not only by their capacity of differentiation, but also by their immunomodulatory properties.

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Isolation of MSC has been reported from several tissues, but since bone marrow derived MSC are the most commonly used cells in clinical trials, these cells probably will be the main target of further studies, in addition an important control for other MSC sources Bone Marrow mesenchymal stem cells obtainment for research is complex, due to the harvest procedure morbidity. In this study, we investigated the possibility of MSC isolation from washing the bone marrow disposable and reusable collection sets. The isolated cells were characterized according to ISCT criteria. There was obtained 3 samples from disposable and 3 samples from reusable collection set. All the samples obtained from bone marrow disposable set successfully derived MSC, but only 2 obtained from the reusable set effectively derived MSC. Despite the fact that reusable filter sets presented smaller pore size which should retain a higher number of cells, we were able to acquire more cells from disposable filter sets. Possible, due to the fact that MSC are plastic adherent and disposable filter sets are enclosed in plastic, in addition to larger areas to be washed. Therefore, this study presented evidences that MSC can be derived from reusable collection kits, which are usable for research as a very ethical source.

Poster Board Number: T-2221

JAGGED-1 INTRACELLULAR DOMAIN EXPRESSED IN MESENCHYMAL STROMAL CELLS SUPPRESSES THE SUPPORT OF HEMATOPOIETIC STEM/ PROGENITOR CELLS IN-VITRO

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In-vitro expansion of human hematopoietic stem and progenitor cells (HSC) is an active area of research. Promising candidates for in-vitro expansion in co-culture systems are mesenchymal stromal cells (MSC). Among a plethora of signalling and structural molecules provided by MSC are ligands of Notch receptors. Immobilized on the surface they were shown to enhance HSC self-renewal in-vitro. In our study we examined if human bone marrow-derived MSC over expressing Jagged-1 could be more effective in the in-vitro expansion of HSC providing Notch ligands simultaneously with molecules physiologically expressed by MSC. Therefore, human bone marrow-derived MSC were transduced with a lentiviral construct containing the open reading frame of human Jagged-1 (Jag-1 MSC). To distinguish the effects associated with Jagged-1 protein itself and Notch pathway activation we also generated cells co-expressing Jagged-1 and the dominant negative form of mastermind1 (Jag-1/dnMAML1 MSC). Over production of Jagged-1 protein was confirmed with immunostaining and Western blot. Functional activity of Jagged-1 and dnMAML1 was proved by expression analyses of Notch target genes, Hes-1 and Hey-1. Next, genetically modified primary MSC were used as feeder cells for short-term (1 week) culture of CD34+ cells from G-CSF mobilized peripheral blood. Surprisingly, a lower number of CD34+ and CD133+ cells was obtained in co-cultures on Jag-1 and Jag-1/dnMAML MSC than on control-transduced MSC. We suppose that Jagged-1 protein itself could modulate HSC supportive potential of MSC. With Western blot analysis of total cell lysate from Jag-1 MSC we determined three fractions of Jagged-1 protein; these are: full length Jagged-1, C-terminal fragment of Jagged-1 and, as we supposed, Jagged-1 intracellular domain (JICD). Thus, we hypothesised that impaired HSC support by Jag-1 MSC could be associated with formation of JICD translocating to the nucleus and influencing gene expression. To confirm our hypothesis, we generated primary MSC over expressing JICD (JICD MSC). Western blot analysis of total cell lysate from JICD MSC revealed the protein being recovered at the same level as the supposed JICD fragment in Jag-1 MSC. Immunostaining of JICD MSC showed a predominant intranuclear localisation. Additionally, some HSC supportive genes, Angiopoietin-1 and SDF-1, were down-regulated in Jag-1, Jag-1/dnMAML and JICD MSC in comparison with control-transduced MSC. This could explain, at least partially, the impaired support of HSC by Jag-1 MSC. In summary, MSC over expressing Jagged-1 protein do not support in-vitro expansion of HSC and this is probably due to formation of JICD and its influence on regulation of gene expression, including HSC supportive genes. These findings require further investigations to understand which role the Jagged-1/JICD pathway may play in human MSC/osteoblasts and which effect it has on the regulation of HSC self-renewal and differentiation in-vivo.

Technologies for Stem Cell Research

Poster Board Number: T-2222

AUTOMATED ANALYSIS AND SORTING OF HUMAN INDUCED PLURIPOTENT STEM CELL (HIPS) CLUSTERS USING LARGE PARTICLE FLOW CYTOMETRY.

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Biomedical methods based on stem cells are currently one of the most dynamic areas in life science and biomedicine. Of particular importance is the development of cellular test systems for disease modeling and pharmacological and toxicological screenings. The reprogramming of somatic cells into human induced pluripotent stem cells (iPS cells) has opened unique perspectives for producing disease and patient specific human cell products. To take full advantage of this technology there is a need (i) to produce a high number of iPS cell lines using high throughput techniques, (ii) to standardize the respective protocols and (iii) to deliver fully characterized cells. Accordingly, the StemCellFactory project aims to fully automate by robotics (i) the generation of human iPS cell lines and (ii) the differentiation of iPS cells into cardiomyocytes and neuronal cells. Currently, standard procedures for iPS cell production are mainly based on manual processing, but automatic procedures are now being developed. This includes, for example, cell pickering devices that recognize and transfer iPS cell and/or clusters from a culture plate to another based on morphology. So far one limitation of this approach is that selection of iPS cells and/or cell clusters requires operator intervention and does not occur automatically. Alternative approaches use immunomagnetic beads and column-based selection of single cells, which however results in loss of iPS cell clonality. We have explored a third approach, one using Union Biometria's large particle flow cytometry technology (COPAS and BioSorter). This technology offers a high throughput technique, which is fully automatable and capable of selecting and sorting cell clusters. The results of our analysis are provided. Human iPS cell colonies were stained with the FITC-labeled pluripotency marker TRA-1-60, collagenase treated and cell clusters were subjected to sorting by flow cytometry with the COPAS device. Cell clusters were sorted according to size and TRA-1-60 expression and distribution in clusters, and single cell clusters were deposited in 96 well format. The analysis was fast and dispensing to wells of multiwell plates was automated. Sorted cells were efficiently expanded as clonal iPS cell lines and used in further studies. This establishes Biometria's

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large particle flow cytometry technology as a versatile device for analysis and sorting of iPS cell colonies.

Poster Board Number: T-2223

ENCAPSULATION IN MONODISPERSE HYDROGEL MICROSPHERES ENABLES FAST AND SENSITIVE PHENOTYPIC ANALYSIS USING LARGE PARTICLE FLOW CYTOMETRY

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Detection and characterization of microorganisms usually involves culture during more than 20 generations in order to achieve the formation of macro colonies on solid media. Alternatively, microencapsulation allows the detection of microbial growth by monitoring the development of micro colonies from encapsulated individual cells. Microbial proliferation inside the microcapsules can be detected using COPAS™ large particle flow cytometry. Here we show the successful application of the Flow Focusing® technology to the microencapsulation of different types of cells in monodisperse hydrogel microspheres. Using a Cellena® Flow Focusing microencapsulator, we managed to produce monodisperse alginate microparticles containing individual bacteria, yeast and human stem cells. Alginate particle sizes were reproducibly selected from less than 100 µm to over 600 µm, by just replacing the disposable nozzle. Sterility was preserved during the microencapsulation procedure, preventing undesired contaminations. Microencapsulated microorganisms were utilized for a variety of applications: from characterizing secreted enzymes to detection of thermosensitive mutants. Proliferation inside the particles was monitored by COPAS large particle flow cytometry without requiring fluorescent labeling.

Poster Board Number: T-2224

SIMULATED MICROGRAVITY INITIATES A CHANGE IN CYTOSKELETON TO PROLIFERATE HUMAN MESENCHYMAL STEM CELLS.

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Physical stimuli such as gravity, electrical field, and magnetic field *etc.*, are common environments during human evolution, which has gone through about 3.5 billion years. The 3D-clinostat, simulated microgravity machine, produced by Mitsubishi Heavy Industries, Ltd., is a multi-directional gravity device, by controlled rotation of two axes, and makes 10^{-3} G average over time (patented: undifferentiated pluripotent stem cell proliferation/differentiation regulation method and system, Japanese patent, publication number P2001-197182A, date of filing June. 28, 2001, P2003-9852A, date of filing January. 14, 2003, and overseas patents, WO2004/061092 A1 PCT [U.S.A., Canada, China, and Korea], P/E [Italy, U.K., Sweden, Germany, and France], 2004). We reported simulated microgravity, one of physical stimuli, inhibited cell differentiation such as myoblasts, osteoblasts, bone marrow stromal cells, and stem cells. Therefore, simulated microgravity is considered as safety physical stimulation for stem cell proliferation. However, it is unclear why stem cells can proliferate or maintain stemness in microgravity environment than in normal 1G environment. We examined human mesenchymal stem cells (hMSCs) cultured in 1G environment and simulated microgravity environment. The number of hMSCs expressed surface markers increased in microgravity. DNA microarray showed the hMSCs in microgravity showed different gene expression, such as cytoskeleton and metabolism, compared to the hMSCs in 1G. The lower expressing genes in microgravity were mostly related to cytoskeleton component. Moreover, hMSCs in microgravity stained with vinculin and F-actin stress fiber showed decreasing number of focal contact and stress fiber network. Focal contact is possibly considered as gravity sensor in space biology. These results suggested the cytoskeleton changing in hMSCs is associated with stem cell proliferation and differentiation in simulated microgravity. Microgravity can provide us novel technology into stem cell biology.

Poster Board Number: T-2225

SYNTHETIC SURFACE FOR CULTURE OF HUMAN BONE MARROW- DERIVED MESENCHYMAL STEM CELLS IN A DEFINED AND XENO-FREE MEDIUM

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Mesenchymal stem cells (MSCs) are multipotent stem cells with the ability to differentiate into bone cells (osteocytes), cartilage cells (chondrocytes) and fat cells (adipocytes). MSCs are an important tool in regenerative medicine and tissue engineering, the therapeutic potential of these cells is being evaluated for several disorders. *Ex vivo* expansion of these cells requires either bovine serum containing media or coating of the culture vessel with human or animal-derived extracellular matrix (ECM) protein. Growing concerns about introducing human and animal-derived pathogens into the culture necessitate the need for an animal free (defined as xeno-free and human origin components-free) culture environment. Also, media components and coating matrices of biological origin may have batch to batch variability and can be undefined. Self-coating requires additional time, resulting in coated vessels with limited shelf-life. Here, we report BD PureCoat™ Fibronectin peptide surface, a synthetic peptide surface for culture of cell types that require Fibronectin coating. The peptide surface is a pre-coated, synthetic, xeno-free, human origin components free, and room temperature stable surface. In this study human bone marrow derived mesenchymal stem cells were cultured on BD PureCoat Fibronectin peptide surface for 5 passages in MesenCult, a defined and xeno-free media. Cell growth and morphology were comparable to cells grown on an ECM coated surface. During the course of culture cells retained their differentiation capability and were successfully differentiated into osteocytes and adipocytes following multiple passages. Expression of MSC markers was determined by flow cytometry. Immunophenotyping revealed that the MSC population was positive for CD90, CD105, and CD73 markers. Cells did not express CD34, CD11b, CD19, CD45 and HLA-DR and stained negative for these markers. BD PureCoat Fibronectin peptide surface provides a ready to use alternative to Fibronectin coating for cell culture with comparable cell attachment and functionality.

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Poster Board Number: T-2226

ISOLATION AND CHARACTERIZATION OF HUMAN FETAL CARTILAGE DERIVED PROGENITOR CELLS.

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Introduction: Bone marrow-derived mesenchymal stem cells (MSCs) are widely used for cartilage tissue engineering but they have also limitations of low differentiation capacity and dedifferentiation *in vitro*. Many studies reported that fetal progenitor/stem cells (FP/SCs) present in various fetal tissues had higher proliferation capacity and differentiation ability than adult stem cells. In this study, we isolated human fetal cartilage-derived progenitor cells (FCPCs) and investigated their characteristics in comparison with MSCs. Materials & Methods: Cartilage tissues of 12-week fetus were obtained after legal abortion with written consent of donors. It was also approved by the Ethics Committee at Inha University Hospital. Cartilage tissue was first washed in PBS containing 1% penicillin/streptomycin. Then, it was minced and treated with 0.1% collagenase type 2 (Worthington Biochemical) in serum free Dulbecco's Modified Eagle's Medium (DMEM) for 16 hrs at 37°C under 5% CO2. Isolated cells were plated at 6000 cells/cm2 and cultured in basal medium (α-MEM containing 10% FBS and 1% penicillin/streptomycin) at 37°C under 5% CO2. Cells were cultured until passage 10, while proliferation rate of cells were measured by doubling time following formula DT= {(T-T0) log2} / (logN-logN0). T-T0 is culture period, N is final cell number, N0 is initial cell number. Differentiation of FCPCs into chondrogenic, adipogenic and osteogenic lineages were examined by safranin O, Oil-red O and Alizalin red S stains after 3 weeks of differentiation *in vitro*. FCPCs were also analyzed for the expression of various cell surface markers for MSCs (Stro-1, CD29, CD44, CD90 and CD105), hematopoietic stem cells (HSCs) (CD34 and CD45), and embryo stem cells (ESCs) (SSEA4/SSEA1, Oct4, Nanog, and Sox2)., In addition sorted by FACs using STRO-1, CD14, CD49C, CD49F, CD49E, CD133, CD166 makers to isolate small size populations(SP). Results & Discussion: We found that cells in basal medium maintained doubling time of less than 3 days and original morphology until passage 10. Differentiation ability of FCPCs was higher than human MSCs for adipogenesis, osteogenesis, and chondrogenesis, which was maintained well until passage 10. FCPCs showed expression of some of MSCs markers and ESCs markers such as CD44, CD90, SSEA4 and Oct4. In the cell sorting experiment, subpopulation of FCPCs with small size populations (SP) were isolated and showed higher expression of CD49f and CD133 than large populations (LP). These results showed that human FCPCs are highly potent cells with unique stem cell characteristics and could be a promising cell source for cartilage tissue engineering.

Poster Board Number: T-2227

TRANSPOSON-MEDIATED BAC TRANSGENESIS IN HES CELLS

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Development of efficient methods for genetic modifications of human embryonic stem cells (hES) is a prerequisite for clinical investigations and fundamental studies of gene function. Large-size transgenes, such as BACs (bacterial artificial chromosomes), that contain distant regulatory sequences, offer an advantage for functional studies, because they can reproduce physiological regulation of gene expression. However, according to our experimental data, the only method published for BAC delivery into hES via nucleofection, leads to the integration of smaller BAC fragments to genomic DNA, which become often silenced. We developed a method for BAC transgenesis in hES cells. The inverted repeats from PiggyBac or Sleeping Beauty (SB) transposon were inserted into the backbone of modified human BACs, which contained GFP-reporters for OCT4, NANOG, PAX6 and GATA4 genes, using recombineering. Co-transfection of BACs with the respective transposase (mPBase, hyPBase or SB100xco) expression construct into H7.S6 and H9 hES cells resulted in generation of stable clones. Lipofection as a delivery method maintained intactness of the BAC and application of transposase ensured full size BAC integration. Our experimental design provided an integration of the BAC via the known sequences (Transposon inverted repeats) that allowed to identify the clones with the full-length BAC by simple PCR screen and to find the sites of integration by Splinkerette PCR. In all clones BAC transgenes were flanked by TTAA or TA sequence confirming transposition by PBase or SB100xco respectively. Most of the stable clones contained 1 copy of the BAC as determined by qPCR. We confirmed functional expression of a reporter for pluripotency OCT4-GFP in hES cells. PAX6-GFP and GATA4-GFP lineage reporters were verified after differentiation of the cells into neuroepithelium and definitive endoderm, respectively. Taken together, we report a reliable and elegant strategy for generation of BAC transgenic hES cells suitable for studies of the expressed and non-expressed genes.

Poster Board Number: T-2228

INDUCIBLE OVEREXPRESSION IN STABLY TRANSFECTED HUMAN EMBRYONIC STEM CELLS

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Overexpression of proteins is a valuable tool to study molecular mechanisms of human embryonic stem cell (hESC) homeostasis. Transient overexpression can give answers to many puzzles, but as transfection rates rarely reach 100 % of cells in the population, stable overexpression is preferred. However, hES cells are known to silence the transgenes and to adopt to the overexpressed proteins leading to artificial in-vitro phenomena. Therefore, inducible overexpression could be a method of choice as latent, non-induced transgenes have better chances to remain in potentially active state during sequential cell passages. We set out to find conditions for establishing inducible hESC clones. First, we screened several available transfection methods, including nucleofection (Lonza), and chemical transfection methods with Fugene 6 and Fugene HD (Roche), NanoJuice (Merck), Lipofectamine (Invitrogen) transfec-

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tion reagents in order to find the best method for experiments with hES cells. Then, we screened several available inducible systems in order to find those which provide satisfactory inducibility and low basal expression in human ES cells. We then constructed a series of vectors inducibly overexpressing 3 different isoforms of fibroblast growth factor 2 (FGF2), in particular isoform 1 (31 kDa), isoform 2 (22,3 kDa) and isoform 3 (18 kDa) as stand-alone proteins and fused to GFP. We confirmed that the constructed vectors overexpress the target proteins upon induction with doxycycline. In addition, we developed a PCR-based approach for transfection of hESC cells with combined linear DNA fragments containing both a gene of interest and a gene of a selection marker. We next compared efficiency of establishing stable clones using traditional plasmid-based transfection with linear, PCR generated DNA fragments containing essential parts of the DNA vectors. For that we first derived subclones of hESC stably and constitutively overexpressing tetracycline-responsive transcriptional transactivator rtTA. We found that chemical transfection with Fugene HD and a Tet-On 3G Inducible Expression system, EF1a Version (Clontech) are the methods of choice for derivation of inducibly overexpressing clones of human ES cells. Our data also suggest that combined PCR based linear DNA fragments are superior over plasmid based transfection for establishing bioengineered sublines of hES cells inducibly overexpressing complex transgenes.

Poster Board Number: T-2229

BIVALENT MUC1* LIGANDS FORM A NOVEL AND IMPROVED SYSTEM FOR DEFINED, XENO-FREE CULTURE OF HUMAN ES AND IPS CELLS

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We report that a single growth factor, NM23, enables serial passaging of both human ES and iPS cells in the absence of feeder cells, their conditioned media or bFGF in a fully defined xeno-free media on a novel defined, xeno-free surface. Stem cells cultured in this system express a pattern of gene expression indicating that they are in a more "naïve" state than stem cells grown in bFGF-based media. NM23 and MUC1* growth factor receptor cooperate to control stem cell self-replication. By manipulating the multimerization state of NM23, we override the stem cell's inherent programming that turns off pluripotency and trick the cells into continuously replicating as pluripotent stem cells. Dimeric NM23 binds to and dimerizes MUC1* which stimulates growth and promotes pluripotency. Inhibition of the NM23-MUC1* interaction accelerates differentiation and causes a spike in miR-145 expression which signals a cell's exit from pluripotency.

Poster Board Number: T-2230

NOVEL SYNTHETIC POLYMERS AND XENO FREE SERUM FREE MEDIA FOR HIGHLY EFFICIENT EXPANSION OF HUMAN MSC

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The goal of this project was to identify novel polymeric surfaces for the culture and expansion of mesenchymal stem cells (MSCs) in a highly promising xeno-free, serum-free chemically defined medium (ITRI-SFM) developed by ITRI (Taiwan). Due to their proliferation and differentiation, as well as their function in pro-angiogenicity and immune modulation, MSCs have been used in treating a variety of serious diseases, such as critical limb ischemia and graft-versus-host disease. Although MSCs are mostly cultured in serum-containing media, the cell therapy industry has been seeking to use an animal-free, serum-free media to avoid the production challenges caused by the safe sourcing of animal components and batch variation of the serum. Unfortunately, the use of such media with standard culture plastics leads to poor attachment and poor growth of MSCs. As a result, ITRI initiated an international collaboration, with Altrika Ltd. (UK), to develop new synthetic substrate candidates which allows rapid expansion of MSCs grown in ITRI-SFM. Altrika used its in-house high throughput polymer microarray platform to screen large libraries of custom designed synthetic polymers. Over 1200 polymers and polymer blends were screened for their capacity to immobilize MSCs and about 20 "hit" polymers were identified. These initial results were subsequently confirmed by scaling-up the surfaces via coating the "hit" polymers onto glass coverslips. Structure activity relationship studies (SAR) were run and novel focused libraries were prepared. These focused libraries allowed the establishment of a clear relationship between the structure and composition of these polymers and their performances in terms of supporting the growth of a range of MSCs: bone marrow MSCs (BMSC), adipose tissue-derived stem cells (ADSC) and Wharton's jelly stem cells (Wj cells). Finally, flow cytometry studies showed that the cells grew on these selected surfaces and maintained characteristic MSC phenotypes of surface markers (CD105+, CD90+, CD73+, CD45-, CD34-). These novel synthetic polymers in combination with ITRI-SFM outperform the existing CELLstart/StemPro system for the expansion of MSCs. They are therefore valuable candidates for the industrial scale production of MSCs in xeno-free serum-free chemically defined medium. This represents a major advance in the development of new MSCs based therapies.

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Poster Board Number: T-2231

HUMAN NEURAL STEM CELL FATE CONTROL BY BIOMIMETIC CUES: FROM SINGLE CELL POSITIONING TO MULTICELLULAR ARRANGEMENTS IN DEFINED *IN VITRO* CONDITIONS

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Biomimetic cues are defined as *in vitro* microenvironmental conditions mimicking cell natural niche. In our hands biomimetic cues used for neural stem cell fate control included creation of biofunctional adhesive domains as well as modulation of oxygen tension conditions, influencing cellular epigenome and stimulation of intracellular pathways by small signalling molecules. Neural stem cells derived from Human Umbilical Cord Blood (HUCB-NSC) were directed either for differentiation or reprogramming in defined *in vitro* conditions. Bioactive domains obtained by micro-contact printing or micro-spotted technology, with controlled content and geometry served as a template to immobilize neural stem cells to the surface and direct their differentiation. It was shown, that specific, receptor mediated versus electrostatic interactions on the cell membrane/bioactive domain interface were crucial to keep the cells either in neurally committed or non-differentiated stage by fibronectin or poly-l-lysine pattern respectively. Single cell positioning in contrast to multicellular domains further promoted non-differentiated stage of HUCB-NSC Activation of intracellular pathways by signalling molecules (Wnt-3a, CNTF, Jagged, Notch and DKK-1) microspotted with fibronectn as bioactive domains directed differentiation of HUCB-NSC into astrocytic and neuronal lineages, as revealed by immunocytochemical and molecular analysis. Low oxygen tension conditions and epigenetic stimulation with small molecules influencing methylation and acetylation status of the cellular chromatin were essential microenvironmental factors for effective induction of iPS cells from HUCB-NSC by poly-arginine tailed Klf4, Oct4 and Sox2 transcription factors. Our studies reviled that combining biomimetic cues with the proper stimulation of intracellular pathways is decisive for successful reprogramming and differentiation of neural stem cells derived from human umbilical cord blood. Sponsored by grant from Polish Ministry of Scientific Research and Higher Education No 5978/B/PO1/2010/38 and No 2211/BP01/2010/38

Poster Board Number: T-2232

A ROBUST SYSTEM FOR EFFICIENT DIFFERENTIATION OF HUMAN PLURIPOTENT STEM CELLS INTO HEMATOPOIETIC STEM AND PROGENITOR CELLS

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Major advances in differentiating human pluripotent stem cells (hPSCs) to primitive hematopoietic stem and progenitor cells would greatly accelerate hematology research and clinical development. In collaboration with Woods et al., Primorigen has developed a high-efficiency hematopoietic stem cell (HSC) differentiation system that currently yields a) 10-100 fold more hematopoietic progenitor cells (CD45/43+, CD34+) than the publication record, b) a 40-fold increase in the number of hematopoietic cells with an adult cell surface phenotype (CD45/43+, 34+, 38-, 90+, 45RA-), and c) clonogenic progenitors (CFUs) at equivalent numbers as umbilical cord blood CD34+ cells. The system combines a proprietary small molecule based medium with an optimized protocol to achieve striking improvements over existing high yield methods across multiple hPSC lines. The resulting cells also show both lymphoid and myeloid cell differentiation potential, greatly increasing their value both for pre-clinical hematology research, and ultimately clinical development of HSC-based therapeutics.

Poster Board Number: T-2233

UNIVERSAL BANK OF IMMUNE-MATCHED CLINICAL GRADE PLURIPOTENT STEM CELLS AND THEIR DERIVATIVES

Craw, John S.[1], Semechkin, Ruslan A.[1], Agapova, Larisa[1], Ross, Richard[2], Kochetkova, Olga[1], Arnold, Lori L.[3], Kalichman, Michael[4], Semechkin, Andrey Y.[1]

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Creating a bank of pluripotent immune-matched stem cells is an essential precursor to developing stem cell based therapies that are both broadly applicable and economically viable. In this abstract we describe a universal bank of pluripotent human parthenogenetic stem cells for use in clinical applications. Such a bank could be used to create banks of differentiated cells that prima facie immune-match millions of individuals, making cellular replacement therapy a clinical reality. Human parthenogenetic stem cells (hpSCs) have been proposed as an alternative source for cell replacement therapies and are known to behave similarly to conventional human embryonic stem cells (hESC) in their proliferative and multilineage differentiation potential. One advantage of hpSCs is that their derivation does not involve the destruction of a viable human embryo. hpSCs are derived from unfertilized oocytes that have been chemically activated and isolated from the inner cell mass of the parthenogenetic blastocyst. Importantly, hpSC can be made to be homozygous at the human leukocyte antigen (HLA) loci. This HLA-homozygosity significantly reduces immunogenicity and simplifies immune matching in clinical applications. Although there are often hundreds of HLA types in any particular population, the majority of individuals carry at least one of high frequency. Differentiated cells derived from hESC will not immune-match any individuals in the population, unless they are naturally HLA-homozygous, and therefore their use as a cell source requires immune-suppression therapy alongside the replacement therapy. However a bank of HLA-homozygous stem cells and their derivatives consisting of the highest frequency haplotypes will immune-match millions of individuals and therefore could provide an economical and accessible therapeutic treatment paradigm. Here we describe our process of creating a bank of ethically derived, clinical grade (Good Tissue Practice and current Good Manufacturing Practice), hpSC, including the regulatory approvals necessary to derive new lines in California. Our strategy is to build on and expand our existing collection and create cell lines that can be used for the clinical development of cellular replacement therapies. Our current bank consists of ten hpSC lines suitable for research, along with HLA-homozygous neuronal lines and retinal pigment epithelial (RPE) cells.

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Poster Board Number: T-2234

SCALABLE EXPANSION OF HUMAN EMBRYONIC STEM CELLS IN A MICROCARRIER SUSPENSION CULTURE SYSTEM

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Human embryonic stem (hES) cells offer great potential for cell therapy and regenerative medicine due to their pluripotency and high capacity for self-renewal. The challenge is to produce these cells in large enough numbers for such therapies, whilst retaining their characteristics. Historically, hES cells have been grown using largely undefined media on mouse or human feeders in cell culture flasks/dishes - a labour intensive system with limited scalability. We have assessed a microcarrier suspension system for the culture of hES cells. A number of hES cell lines were grown on gelatin, laminin or Matrigel coated microcarriers in the LeviTube, an impeller free vessel for microcarrier and suspension cell cultures. The system supported the growth and serial subculture of all hES cell lines used. Cell yields were increased by up to 25% compared to the same line on inactivated mouse feeders in 6-well plates over a five day period. hES cells were also frozen on the microcarriers and successfully thawed to produce viable cultures. This system, when compared to 2-D culture systems, provides a method for producing the equivalent of 15 to 20 six-well plates in a single 50 mL tube under defined, feeder-free conditions with the key benefits being significant reductions in both handling time and media consumption.

Poster Board Number: T-2235

THE IMPORTANCE OF DIGITAL HOLOGRAPHIC MICROSCOPY FOR AUTOMATED, REAL-TIME MONITORING OF HUMAN ADULT STEM CELL CONFLUENCE IN LARGE-SCALE CULTURES.

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To guarantee robust cell expansion on a large scale, an automated control method becomes essential for obtaining sustainable and useful stem-cell-based products. Given that both stem cell behavior and the differentiation mechanisms are sensitive to cell density, monitoring of cell confluence is mandatory. Actual observation protocols of traditional polystyrene T-flasks, or multitray stacks, are ineffective for large scale manufacturing. Integrity™ Xpansion™ multiplate bioreactors have been developed to enable noninvasive, real-time observation of stem cell growth at large scales. The specific design of the bioreactor combined with the iLine – a differential digital holographic microscope (DDHM) of the newest generation – enables automatic multiplate cell monitoring. The DDHM technology captures 3-D information, enabling label-free image processing and automatic cell confluence counting. The data presented will highlight the benefits of differential digital holographic microscopy as a reproducible and consistent method to track stem cells confluence during large-scale production.

Poster Board Number: T-2236

COMPARATIVE ANALYSIS OF *EX VIVO* EXPANDED HUMAN CD34+ HEMATOPOIETIC STEM CELLS ON NANEX™ NANOFIBER PLATES

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Hematopoietic stem cells (HSC) are increasingly used in treating a wide variety of malignant and degenerative diseases with promising results. Cells expressing CD34 (CD34+) in bone marrow (BM), mobilized peripheral blood (PB) and umbilical cord blood (UCB) identify a rare cell population with HSC progenitor characteristics. The number of CD34+ HSC available is invariably limited and for some clinical applications, *ex vivo* expansion is required to generate the cell numbers needed for successful transplant. The development of *ex vivo* culture systems that enable efficient expansion and maintenance of CD34+ HSC is a crucial step to harnessing its full potential for use in cell-based therapies. Arteriocyte recently launched an *ex vivo* culture system, the NANEX™ HSC Expansion Kit. The NANEX™ coating is a chemically-modified polymeric nanofiber mesh that forms a 3D scaffold upon which the cells adhere. This 3D scaffold partially mimics the BM microenvironment promoting cultures of CD34+ cells on NANEX™ plates to efficiently expand and maintain the CD34+ phenotype. Most of these studies were performed using CD34+-selected cells from UCB. In this study, we independently evaluated Arteriocyte's NANEX™ HSC Expansion Kit as an *ex vivo* culture system using cryopreserved CD34+-selected cells from UCB and mobilized PB cultured in parallel. Three samples each of frozen CD34+-selected cells from UCB and mobilized PB were thawed and cultured in parallel for eight days on NANEX™-coated or regular 6-well tissue culture (TC) plates. The cells were maintained in serum-free HSC expansion medium and optimized cytokine cocktail provided in the kit. Cell counts, flow cytometry analyses and colony-forming cell (CFC) assays were done both at initiation and at termination of cell cultures. There was on average, a 124-fold expansion of UCB CD34+ cells and four-fold expansion of mobilized PB CD34+ cells on NANEX™-coated plates after the eight-day culture period. In contrast, UCB CD34+ cells cultured in uncoated TC plates had a 25-fold expansion and mobilized PB CD34+ cells did not proliferate at all. Flow cytometry analyses of cells harvested from NANEX™ plates showed that UCB CD34+ cells maintained a larger percentage of CD34+ cells (average = 24.5 %) compared to mobilized PB CD34+ cells (average: 9.7 %). UCB and mobilized PB CD34+ cells harvested from uncoated TC plates had markedly lower percentages of CD34+ cells (8.5 % and 5.0 %, respectively). There was no significant difference in CFC content (CFC per 500 CD34+ cells plated) between CD34+ cells used to initiate the cultures and the cells harvested after the eight-day culture period, indicating that NANEX™ culture maintains colony-forming cells. This study validates the use of NANEX™ HSC Expansion Kit as an efficient system for the *ex vivo* expansion of UCB CD34+ cells. Expansion of mobilized PB CD34+ cells was likewise enhanced but not to the same degree. Mobilized PB CD34+ cells are known to be less proliferative than UCB CD34+ cells, and efforts to expand these cells *ex vivo* have not been as successful to date. It is likely that mobilized PB CD34+ cells might require other growth factors and/or interactions with the microenvironment for their optimal growth. Nonetheless, the results of these initial *ex vivo* experiments look promising and further studies are warranted.

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Poster Board Number: T-2237

RECOMBINANT HUMAN VITRONECTIN AS A COST-EFFECTIVE XENO-FREE SUBSTRATE FOR EXPANSION OF HUMAN PLURIPOTENT CELLS AND DIFFERENTIATION OF ENDODERMAL AND ECTODERMAL LINEAGES IN 2D AND 3D

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Previously we reported that Primorigen's engineered version of xeno-free recombinant human vitronectin (rhVitronectin(TM)) supports pluripotent cell growth and directed differentiation of iPS cells into endodermal lineages in 2D culture. We now have demonstrated the same capabilities for iPS expansion and endodermal differentiation in 3D microcarrier culture, and ectodermal differentiation in 2D. Primorigen's rhVitronectin contains the entire mature human vitronectin sequence and possesses key advantages over human-plasma derived vitronectin, including reduced costs, reduced batch-to-batch variability, and reduced risk of adventitious agents. These properties are particularly important for therapeutic applications of stem cells, given the growing interest in expansion, propagation, and differentiation under completely defined and xenobiotic-free conditions. Such applications potentially could be extensive, as Vitronectin is a well-known component of the extracellular matrix that supports cell adhesion and spreading, and has been shown to support ES cell expansion (Braam et al 2008) and iPS cell expansion and differentiation (Rowland et al 2010). Moreover, the vitronectin receptor, Integrin alphaV, is expressed by pluripotent cells, by migrating mesodermal cells during gastrulation, in hES derived definitive endoderm, and in many adult differentiated cell types including astrocytes and keratinocytes [ectodermal], osteoclasts and lymphoid blood cells [mesodermal], and hepatocytes and pancreatic beta-cells [endodermal]. Thus rhVitronectin is likely to enable cellular adhesion of derivatives from all three primitive germ layers during *in vitro* directed differentiation protocols.

Poster Board Number: T-2238

HYPOXIC CULTURE OF HUMAN PLURIPOTENT STEM CELLS IS PERMISSIBLE USING MOUSE EMBRYONIC FIBROBLASTS

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Introduction:
Human pluripotent stem cells (hPSCs) such as embryonic (hESCs) and induced pluripotent stem cells (iPSCs) are capable of producing dopaminergic (DA) neurons that could be used to treat patients suffering from Parkinson's disease. Hypoxic culture of hPSCs is utilized to mimic the in vivo environment surrounding the pre-implantation embryo, where undifferentiated hESCs are isolated. This environment typically ranges from 1.5 - 5.3% O2 between different mammalian species, which is substantially lower than atmospheric oxygen tension (~20%). When cultured in hypoxia, hESCs and hiPSCs have enhanced proliferative capacity and enhanced potential for neuronal differentiation, producing increased yield of target neurons. Most published works culturing hPSCs under hypoxia use costly feeder-free culture systems that are limited by problems associated with cell instability, poor cell attachment and acquirement of chromosomal abnormalities. This work examines the effects of hypoxia on the traditional co-culture system of inactivated mouse embryonic fibroblasts (iMEFs). We looked to determine whether iMEFs are compromised by hypoxia in a way that may affect their ability to produce growth factors required to maintain hPSC self renewal and pluripotency, or whether it is permissible to use iMEFs during hypoxic culture of hPSCs. Results: We demonstrated that under hypoxia, iMEF viability and metabolic activity is not significantly altered over 7 days when compared to the normoxic control. In addition, there was no significant difference in the consumption or production of media components or metabolites in iMEFs cultured under hypoxia or normoxia. Furthermore, there was no difference in growth rate of colonies cultured in hypoxic versus normoxic culture conditions in hESCs. Cells cultured in hypoxia for over six months still expressed pluripotency markers Tra1-60 and Oct4, indicating that pluripotency of hPSCs are maintained under hypoxic culture utilising iMEFs. To verify that hypoxic conditions were attained, nuclear accumulation of HIF1α was confirmed in iPSC cultured in hypoxia for 6 hours by immunostaining, and transient expression of HIF1A in cells cultured for up to 7 days in hypoxia was demonstrated by western blotting. Conclusions and Future Work: We conclude that hypoxia does not significantly affect viability or metabolic activity of feeder cells, nor is it detrimental to hPSC pluripotency. Our ongoing work addressing DA neuronal differentiation of hPSCs therefore adopts a hypoxic culture system using iMEF feeders. In our current research, we are assessing the effect of overexpression of Lmx1a, Pitx3 or FoxA2 on acquisition of a DA neural phenotype in hypoxic culture conditions. We hypothesise that overexpression of these markers of DA lineage, coupled with the positive effects of hypoxia, will enhance the yield and purity of DA neurons.

Poster Board Number: T-2239

HUMAN BASEMENT MEMBRANES IN XENO-FREE CULTURE SUPPORT MRNA-REPROGRAMMED HUMAN INDUCED PLURIPOTENT STEM CELLS

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Basement membrane-rich Matrigel® is a proven feeder-free substrate for the propagation of induced pluripotent stem cells (iPSCs) *in vitro*, yet the xenogenic origin of Matrigel® is problematic for iPSC clinical translation. Here we describe human basement membranes (HBMs) derived from allogeneic tissues for iPSC culture and therapeutic delivery. HBMs share a protein composition similar to Matrigel® in terms of laminin, collagen IV, HSPG, and nidogen content as shown by western blotting and MALDI-TOF mass spectrophotometry. Further, HBM can be synthesized into three dimensional electrospun nanofibers and sponges, forming scaffolds that are implantable *in vivo* which we have characterized *in vitro* as cell delivery vehicles. For iPSC culture, a wet and dry coating method was formulated to attach the HBMs to tissue culture plastic, with Bradford assays showing equivocal protein coating between HBMs and Matrigel®. In serum-free attachment assays, HBMs, both as films and nanofibers, showed strong stem cell binding kinetics. In an entirely xeno-free system, we show that HBMs can be used to attach, propagate, and maintain the pluripotency of non-integrating mRNA generated iPSCs made from consented tissues, with pluripotency validated by TRA-1-60, TRA-1-81, SSEA-4, Nanog, and Oct-4 immunohistochemistry done throughout extended passages. Production of human basement membrane as a xeno-free culture substrate takes an important step in advancing iPSCs towards clinical translation. The ability to tissue engineer HBM further offers a myriad of potential regenerative medicine applications for this novel allogeneic biomaterial.

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Poster Board Number: T-2240

NON-INVASIVE DETECTION OF MODIFIED MOUSE AND HUMAN HEMATOPOIETIC STEM CELLS DURING IMMUNE CELL DEVELOPMENT WITH A HUMANIZED PET REPORTER GENE AND [18F]-L-FMAU

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Positron Emission Tomography (PET) is a quantitative whole-body clinical imaging modality which utilizes radiolabeled probes to detect specific biological processes *in vivo*. PET reporter genes (PRGs) non-invasively monitor the location of transplanted cells carrying the gene *in vivo* by imaging with a reporter specific probe. Current clinical PET reporters are derived from Herpes Simplex Virus Type I thymidine kinase (HSV1-Tk) which is immunogenic and has led to the elimination of therapeutic cells. Transgenes of human nucleoside kinases are potentially non-immunogenic alternatives to viral-based PET reporters. We tested whether a human deoxycytidine kinase with three point mutations (hdCK3mut) could monitor therapeutic regeneration of the hematopoietic system in mice transplanted with modified hematopoietic stem cells (HSCs) of mouse (m) and human (h) in xenotransplantation protocols. hdCK3mut provides a broader substrate capacity accommodating thymidine derivatives like [^{18}F]-L-FMAU which was tested as a potential PET reporter probe. *In vitro* murine bone marrow colony assays determined that hdCK3mut labeled cells retain equivalent colony forming capacity compared to non-transduced bone marrow. *In vivo,* serial [^{18}F]-L-FMAU PET analysis of hdCK3mut labeled mHSC recipient animals over 32 weeks depicted the dynamics of hematopoietic reconstitution visualizing the successive homing of mHSCs to different hematopoietic tissues in the murine system. hdCK3mut retroviral vector could be detected in all major lymphoid and myeloerythroid cell types within multiple hematopoietic tissues by FACS and IHC. Continuous long-term expression of hdCK-3mut PRG caused no observable deleterious effects as determined by normal engraftment in peripheral blood analysis. No survival advantage/disadvantage was detected in mice transplanted with hdCK3mut mHSCs compared to normal mHSC recipient animals. We determined that hdCK3mut was capable of detecting hHSC engraftment in mice providing an innovative tool to monitor human xenotransplantation protocols. Both models demonstrated that hdCK3mut causes no detrimental effects in HSC differentiation and is retained *in vivo* with no counter selection. hdCK3mut is a potentially non-immunogenic human based PET reporter gene that can be broadly useful for monitoring the long term outcomes of clinical regenerative therapies.

Poster Board Number: T-2241

AN *IN VITRO* ASSAY FOR CLONOGENIC, HIGH-THROUGHPUT ANALYSIS OF MURINE INTESTINAL STEM CELLS

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Recent advances in the isolation and culture of intestinal epithelial stem cells (IESCs) facilitate testing functional properties of stemness *in vitro*. To establish 'stemness' of putative IESCs that are defined by restricted gene expression signatures, single cells must demonstrate multipotency and self-renewal. Although current *in vitro* assays support growth of IESCs, they are not amenable to efficient clonogenic analysis at the single cell level. Additionally, limitations of the current assay make IESC-derived enteroid structures (bodies of crypt/villus-like units) difficult to process for downstream molecular analysis. The development of micro-fabricated cell culture arrays and computational image analysis provides the next technological step toward a clonogenic assay for IESCs and other somatic stem cells. Here, we show that single Sox9low-expressing IESCs can be seeded into cell culture microwell arrays and develop into enteroids, exhibiting the same behavior as IESCs in conventional *in vitro* assays. Single IESCs can be separated into distinct microwells, facilitating day-to-day quantification of clonogenic development. We demonstrate that this microwell array technology enables efficient clonogenic analysis and provides a platform for powerful statistical analysis of the effects of small molecules on IESCs. Support: NIH R01 DK091427, NIH R03 DK089126, NC Biotechnology Center Grant.

Poster Board Number: T-2242

RADIATION EXPOSURE ON MOUSE AND HUMAN INDUCED PLURIPOTENT STEM CELLS ENABLES REMOVAL OF TERATOMA-FORMING CELLS IN THE TRANSPLANTATION OF DIFFERENTIATED CELLS

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There are problems that tumor-like cells appear in transplantation therapy using iPS cells (iPSCs). Contamination and proliferation of undifferentiated cells that do not respond to differentiation induction are thought to cause tumorigenesis. The undifferentiated cells are generally sensitive to radiation exposure. We performed an experiment to test the hypothesis that the irradiation selectively removes undifferentiated iPSCs which heterogeneously coexist in differentiated cell population. Colony forming abilities were investigated by various irradiation doses in mouse iPSCs (miPSCs, MEF-Ng-20D-17) and human iPSCs (hiPSCs, 201B7). The irradiation inhibited colony formations in miPSCs (8 Gy) and hiPSCs (4Gy),

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however, it had no effects on the feeder cells (fibroblast cells). Next, miPSCs and hiPSCs were induced differentiation into cardiac muscles, neurocytes and other differentiated cells according to conventional methods. As markers of coexisting undifferentiated cells by flow cytometry analysis, GFP positive (Nanog genes expression) cells were examined for miPSCs and anti SSAE-4 antibody were for hiPSCs. In miPSCs, mean ratios of GFP positive cells were 14 % in embryoid bodies and decreased to 4 % in 4 weeks differentiation induced cells. In hiPSCs, mean ratios of SSEA-4 positive cells were 22% in 4 weeks differentiation-induced cells and decreased to 4% in 8 weeks cells. We tried to eliminate undifferentiated miPSCs on the culture dishes by irradiation. By a single exposure dose of 8 Gy to the culture dishes, GFP positive cells were decreased to lower than 0.1 % without disorder in myocardial beats. As *in vivo* studies, we injected non-irradiated or irradiated differentiated cells into the testis of SCID mouse to examine teratoma formations. Although non-irradiated cells formed teratomas in all the mice examined (n=13), irradiated cells formed either no or localized teratoma in the testis (n=20). Therefore, it was suggested in the miPSCs that the irradiation before transplantation selectively eliminates residual undifferentiated cells and inhibits post transplantation tumorigenesis. In hiPSCs, the irradiation also inhibits teratoma formations by an appropriate amount of dose since coexistence of undifferentiated cells was observed in the differentiation induced cells.

Poster Board Number: T-2243

A NOVEL METHOD FOR INDUCING A CELL TYPE-SPECIFIC CRE-LOX RECOMBINATION IN MOUSE ADULT BRAIN STEM CELLS

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One of the major challenges of brain tumour research remains the precise and selective targeting of specific cells in neurogenic regions. These cells, thought to be the origin of most intrinsic brain tumours, reside in the subventricular zone, one of the major neurogenic areas of the brain.
Here we demonstrate a novel approach in which we target the B-Type of SVZ cells, by combining a cell type-specific glutamate aspartate transporter (GLAST)-Cre ERT2 mouse model with the intraventricular injection of a recombination inducer- the active Tamoxifen metabolite. Our new technique is also compared to the previously reported method using Adeno Cre virus. With this novel approach we aim at selectively targeting the SVZ B-type of cells. Mice expressing the CreERT2 fusion protein under control of the Glutamate-aspartate transporter promoter express cre in astrocytes including B-Type SVZ stem cells. Tamoxifen administration induces nuclear transfer of the cre-ERT2 protein in GFAP-expressing cells, where it can mediate loxP-dependent recombination. GLAST-CreERT2 mice were crossed with a LacZ reporter mouse (ROSA26RloxP/loxP) which allows detection of a recombination with the β-galactosidase assay. We administered the metabolically active Tamoxifen derivative by injection into the ventricle of GLAST-CreERT2 x ROSA26RloxP/loxP mice. Identification of the recombined cell types was confirmed by immunohistochemistry or by β-galactosidase assay, and by colocalisation with GFAP, Nestin, Sox2, PDGFRα or Doublecortin. Direct injection of the Tamoxifen metabolite into the ventricles of GLAST-Cre mouse brains induced cre mediated recombination. Recombination occurred in GFAP positive cells in the SVZ, and at higher doses, occasionally also in adjacent differentiated astrocytes. The co-expression of the SVZ stem cell marker GFAP and to a much lesser extent, the progenitor marker Nestin in recombined SVZ cells proves the efficiency of the spatially- and temporally-controlled recombination of GLAST-positive cells with a stem cell phenotype. Our approach demonstrates the selectivity of the method that makes it a very useful model for targeting SVZ-stem cells in the context of our mouse models of brain tumour pathogenesis.

Poster Board Number: T-2245

IMPORTANCE OF SIZE DIFFERENCE BETWEEN MACRO- AND MICRO-SCALE STATIC CULTURES TO ENABLE EFFECT OF CELL-SECRETED FACTORS ON MOUSE EMBRYONIC STEM CELL BEHAVIOR - A MATHEMATICAL MODEL BASED STUDY

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Conventional static macro-scale cultures, e.g. 6-Well plate (6WP), cannot fully realize effect of cell-secreted factors owing to their large culture volumes, and distribution of the factors from cell neighborhood by spontaneous fluctuations causing a high mass transfer rate. To implement cell-secreted soluble factors for auto-regulation of ESC (embryonic stem cell) behavior in culture, we cultured and differentiated mouse ESC (mESC) on a semi-permeable membrane sandwiched between two micro-chambers (height 500 μm) of a microbioreactor (MB). During cell culture in the MB, we maintained small culture volume (~114 μL) in the top chamber to form enclosed diffusion-dominant environment while culture volume in the bottom chamber was adjusted to provide enough nutrient. Recently, we showed that cell-secreted BMP4 in the MB co-operated with LIF - but not in the 6WP - to preserve a high pluripotent state of mESC culture. We also showed that cell-secreted BMP4 induced prominent meso- and endo-dermal differentiation of mESCs in the MB culture instead of default neuronal differentiation usually observed in 6WP owing to absence of signaling other than FGF. Therefore, MB realized action of cell-secreted soluble factors which cannot be realized in macro-scale cultures and provided an environment which enhanced ESC self-capabilities by auto-regulation. Owing to a smaller volume than the macro-scale one (6WP), concentration gradient along the height of the MB will be relatively small causing a high availability of cell-secreted BMP4 to mESC in the MB culture. In addition, we observed slower dye dispersion in the MB than the 6WP system without cell indicating slower mass transfer rate in the MB. This can also cause a high availability of BMP4. Overall, higher availability of cell-secreted BMP4 than the 6WP can activate autocrine/paracrine effect to enable a high pluripotent state or different differentiation profile in the MB. However, we did not know which mechanism (small concentration gradient or slow mass transfer rate) difference between the MB and 6WP had prominent role to differ the availability of BMP4. In this study, we developed mathematical models to estimate cell-secreted BMP4 activities in the MB and 6WP cultures to identify the prominent mechanism difference. Owing to heterogeneity of differentiated cells in differentiation culture necessitating a large number of parameters to estimate BMP4 activity, we only considered undifferentiation culture of mESCs. The models quantified autocrine activity of BMP4 (proportional to concentration of BMP4-bound receptors) in the culture systems by considering diffusion of BMP4 in culture volume; diffusion and secretion of BMP4, binding of BMP4 to its receptor, etc. in mESC aggregates. Models estimated higher concentration of BMP4-bound receptors in the MB culture than the 6WP indicating enhanced autocrine activity in the MB. By varying culture volume height (to reflect the role of concentration gradient) and diffusion constant (to reflect the role of mass transfer rate), it was found that height difference would cause larger variation in autocrine activity between the MB and 6WP than mass

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transfer difference would. Therefore, smaller concentration gradient i.e. smaller size of the MB instead of slower mass transfer rate than that of the 6WP would have the prominent role to activate effect of cell-secreted BMP4 on mESC behaviors in the MB.

Poster Board Number: T-2246

IDENTIFYING SHARED HUMAN AND MOUSE STEM CELL PATHWAYS ASSOCIATED WITH LEUKEMIA STEM CELLS: STANDARDIZING COMPARISONS USING THE HSCI STEM CELL COMMONS

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The weight of evidence indicates that tumor propagation depends on a subpopulation of tumor-initiating cancer stem cells (CSC) endowed with true self-renewal capacity. It is attractive to target pathways to cancer progression through selective action on CSCs. Leukemia stemness signatures have been defined using functionally well defined leukemic stem cells. Although these signatures share a number of significant oncogenic pathways, the signatures only have three genes in common. Stem cell molecular and experimental comparison is thrown into sharp relief by inconsistencies in actual results, data formats, and descriptions among biomedical research discoveries. The Harvard Stem Cell Institute (HSCI) has created an open, community-based approach to data sharing: The Stem Cell Commons (stemcellcommons.hsci.harvard.edu) that captures information in an Investigation-Study-Assay (ISA) format used by over 30 organizations. The common framework means that workflows and methods are consistent, and that experiments can be matched across 148 studies using common experimental descriptions. To further address inconsistency and find key activating signaling pathways in CSCs, we have developed pathway fingerprinting, a method that standardizes gene expression to a fixed set of curated pathways (KEGG, Reactome and Wikipathways). Pathway fingerprinting out-performs gene-based approaches for cross-experiment analysis, permitting comparisons of experimental results independently of technology, species or experimental source and providing a simplification of the cellular functional state. We have pathprinted haematopoetic stem cells in mouse, human and leukemic stem cells in mouse models and human published data. We show that cancer stem cells share pathway activation to recapitulate stem cell ontogeny, sharing immune signaling, hemostasis, and a number of pathways at several levels of functional organization. We welcome participation from the stem cell community and invite researchers to join the Commons to promote discovery in stem cell research by sharing data and tools.

Poster Board Number: T-2247

3D-MODELING OF EMBRYOID BODIES FORMED BY MOUSE PLURIPOTENT STEM CELLS IN DIFFERENT CELL CULTURE SYSTEMS

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Pluripotent stem cells derived from different cell sources can recapitulate *in vitro* the early stages of mammalian development. Despite different origin, embryonic stem (ES) and embryonic germ (EG) cells express similar gene sets and display similar differentiation potentials after the *in vitro* cell culture adaptation. Their malignant counterparts, embryonal teratocarcinoma stem (EC) cells, have restricted developmental potentials caused by genetic disturbances and represent cell models for study of developmental abnormalities. In order to develop standard 3D cell models for fundamental and pharmacological research the spatio-temporal characteristics of the earliest stages of *in vitro* differentiation of embryoid bodies formed by mouse ES, EG and EC cells were studied. We found the significant differences in growth and differentiation dynamics of embryoid bodies formed by these cell lines maintained in serum and serum-free cell culture systems. Similarly, the morphogenetic events timing and early embryonic lineage commitment differed in embryoid bodies formed by ES, EG and EC cells. Moreover, growth and differentiation dynamics of the embryoid bodies also depended on the initial cell numbers forming spheroids. Electron microscopy study and live cell imaging analysis showed that during the differentiation of embryoid bodies the intercellular contacts underwent changes and cell communications between outer and inner cell layers of the embryoid bodies progressively reduced. Therefore, diffusion of high molecular weight substances from culture media and growth factors secreted by outer extraembryonic endoderm cells significantly decreases upon embryoid body differentiation. These findings provide new information for the investigation of 3D differentiation of different types of pluripotent stem cells exposed to xenobiotics, new pharmacological substances and drugs.

Poster Board Number: T-2249

HETEROGENEOUS EXPRESSION OF PLURIPOTENCY-ASSOCIATED GENE IN MOUSE EMBRYONIC STEM CELLS VISUALIZED BY BIOLUMINESCENCE MICROSCOPY

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The embryonic stem (ES) cells consist of various cell subsets which express different levels of pluripotency associated genes such as Nanog, Oct4, and Sox2. The heterogeneous expression of the transcription factors associated with pluripotency within ES cells has been shown by the use of RT-PCR, luciferase reporter assay, flow cytometry, and immunohistochemistry. Although these conventional methods provide the end-point data of gene expression, they cannot monitor the temporal changes within individual cells. Therefore, the temporal and spatial analysis of gene expression at the single cellular level is required for studies on self-renewal and differentiation processes of ES cells. To understand the exact profile of gene expression in the process of ES cell differentiation, gene expression of Nanog in mouse ES cells were monitored by using the single cellular bioluminescence imaging system (LV200, Olympus). The time course imaging analysis using luciferase as a reporter revealed that (1) the promoter activity of Nanog gene mostly fluctuated at single cellular level, and the pattern of fluctuation varied among colonies of ES cells in the presence of leukemia inhibitory factor, (2) the prompter activity of Nanog gene decreased in the most of ES cells after bFGF-induced differentiation, whereas higher Nanog expression was sustained in small subpopulation of ES cells, and (3) the promoter activity of Nanog gene recovered heterogeneously among ES cells in response to dual inhibition of mitogen-activated protein kinase signaling pathway using PD184352 and SU5402. This is the first study to demonstrate the temporal and spatial dynamics of Nanog gene expression at the single cellular level using bioluminescence imaging system.

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Poster Board Number: T-2250

GENERATION OF ROSA26-TDTOMATO KNOCK-IN RATS VIA GENE TARGETING OF PLURIPOTENT STEM CELLS

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Use of rats for studies in behavior, pharmacology, and disease modeling has been limited because gene targeting technology has been lacking. However, recent discovery of a culture system using small molecules specifically to inhibit spontaneous differentiation pathways of pluripotent stem cells (PSCs) has permitted generation of germline-competent rat PSCs. This stable and reproducible culture system in rat PSCs constitutes a breakthrough for generating not only transgenic rats by introducing exogenous genes into PSCs but also knock-out rats via gene targeting. Various genetically modified rats will soon be available for analyses of gene functions or physiological features as can now be done in mice. In mouse or human, one widely used technique to express a gene of interest stably and ubiquitously is to insert that gene into the Rosa26 locus via gene targeting of PSCs. Rosa26 knock-in mice conditionally expressing a reporter or a toxin gene have contributed to tracing or ablation of specific cell lineages. Here, as a third model, we report identification of a Rosa26 locus in the rat. We found a highly conserved region in rat chromosome 4 that contains not only the a Rosa26 locus but also genes that are neighbors to Rosa26 in mouse. To see if this region, like the Rosa26 locus in mouse or human, actually allowed ubiquitous expression of an inserted gene, we cloned homology arms from genomic DNA of the DA rat and constructed a targeting vector to insert a splice acceptor with tdTomato-IRES-Puro^r-pA sequence. After electroporation into rat PSC lines derived from various strains and tissues, on average, about 30% of picked up clones demonstrated correct targeting judged by PCR using genomic DNA and Southern blotting. As expected, all positive clones ubiquitously expressed tdTomato. We injected rat embryonic stem cells (ESCs) from one of the Rosa26-tdTomato knock-in lines into rat blastocysts. Neonatal chimeric rats expressed tdTomato throughout their bodies and they grew into adults normally and showed high coat color chimerism. Next, we mated 3 male rats exhibiting relatively higher chimerism with wild type females to see if Rosa26-tdTomato knock-in rat ESCs were capable of germline transmission. Offspring of 2 chimeras expressed tdTomato ubiquitously throughout their bodies, indicating successful germline transmission. Expression of tdTomato was detected in all main organs at the neonatal stage and in blood cells at the adult stage. These data provided strong evidence for functional conservation of the Rosa26 locus among rat, mouse, and human. We have successfully generated Rosa26 knock-in rats expressing tdTomato ubiquitously via gene targeting of PSCs. The new tools described here (targeting vectors, knock-in PSCs, rats) should be useful for a variety of research using rats.

Poster Board Number: T-2251

THE EXPRESSION OF MULTICISTRONIC VECTORS LINKED BY THE SELF-PROCESSING 2A PEPTIDES IN MOUSE EMBRYONIC STEM CELLS AND THE EFFECTS OF USING DIFFERENT 2A PEPTIDES ON PROTEIN EXPRESSION

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Multicistronic vectors linked by the self-processing 2A peptides have been successfully used in cellular reprogramming. The function and expression characteristics of these vectors have yet to be well documented in embryonic stem cells. In the present study, we generated vectors containing different combinations of 3 pancreatic transcriptions factors (Pdx1, Nkx2.2 and Ngn3) together with an eGFP reporter, all linked by the self-processing 2A peptides. The multicistronic vectors were trasnfected into mouse embryonic stem cells and the expression of these vectors was characterised using qPCR and FACS. Similar level of gene expression of a given gene was observed regardless of its position within a 2A multicistronic construct. Expression of a terminal EGFP was 50.6% and 23.8% more efficient when linked by T2A compared with F2A and E2A peptides, respectively. Highest level of expression was achieved when all genes in a multicistronic vector was linked the genes exclusively with T2A peptides. The study provides guidance on successful strategies for the efficient expression of 2A multicistronic constructs in embryonic stem cells.

Poster Board Number: T-2252

TWO DISTINCT KNOCKOUT MODELS REVEALED AN ESSENTIAL ROLE OF P53 GENE IN RAT EMBRYOGENESIS.

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Rats have important advantages on an experimental system for physiological and pharmacological investigations. However, gene functions *in vivo* have been poorly understood because of lacking knockout (KO) strategy in the rats. In 2008, Zinc-finger nuclease (ZFN)-based KO technology was developed in the rats. The technology makes it possible to generate both KO and knockin (KI) rats more rapidly than a conventional KO method, which suggests a lack of merit using embryonic stem cells (ESCs) to generate KO animals. In this study, we show a combination strategy using both ZFNs and ESCs for the generation of knockout chimeras in the rats. This method allowed us to find a novel phenotype, which was not observed in a conventional KO model, within 1 month since homologous recombination was demonstrated in the ESCs. In the present study, p53 was targeted for generating KO rats to investigate its function on rat development using an Oct4-Venus ESC line that was established in our previous study under a condition of YPAC culture medium composed of 4 signaling inhibitors and 20% serum. ZFNs strongly supported homologous recombination, leading to obtaining not only heterozygous (p53+/-) clones but also homozygous (p53-/-) ones. Chimeric rats were generated via microinjection with p53+/- ESCs and heterozygous rats were produced through germline transmission. An intercross of heterozygous rats resulted in a successful delivery and growth of homozygous males while females did not grow due to causing neural tube defects (NTDs), exencephaly. On the other hand, microinjection with p53-/- ESCs led to a failure of delivery. During their embryogenesis, most of the

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p53-/- chimeras stopped development, resulted in absorption in uteri. An analysis of p53-/- ESCs revealed that they were protected from apoptosis and maintained strong pluripotency under a differentiating condition. Moreover, chromosomal instability appeared in ESC-derived differentiated cells whereas the p53-/- ESCs kept a normal karyotype despite long passages. These results suggest that the malignancies in the p53-/- ESCs might interfere normal embryogenesis in the chimeric environment. Here we conclude that the phenotype by a p53 deficiency was remarkably different between conventional and chimeric KO rat models, which highlighted the chimeric strategy using ZFN-mediated homozygous ESCs to promptly find concealed functions of genes.

Poster Board Number: T-2253

CORRELATION BETWEEN OSTEOBLASTIC DIFFERENTIATION ACTIVITY AND CELL MORPHOLOGY OF RAT MESENCHYMAL STEM CELLS

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Introduction: Cell morphology has received much attention in recent years due to the effect of cellular functions, including proliferation, differentiation and migration, which are important on bioactive molecules. Recent evidence suggests that extracellular environments can also mediate cell functions, particularly cell adhesion behaviors. Tanaka and Hirose et al. reported that rat mesenchymal stem cells (MSCs) with round-shape showed higher ostoblastic differentiation activity on ceramics sheets than those with wide spread-shape on polymer sheets after 2 weeks of cultures with osteogenic media. However, correlation between osteoblastic differentiation activity and cell morphology of rat MSCs remains unclear. In the present study we report the correlation of cell adhesion areas on various substrates and osteoblastic differentiation of rat MSCs. Materials and Methods: Five various substrates were used for MSCs culture in this study. The titanium plates(11mm×11mm×1mm) were immersed in hydrogen peroxide (H2O2), then treated by autoclaving (H2O2-Ti) or heat-treated at 300 ºC for 2 hours (300 ºC -Ti). Ti, H2O2-Ti and 300 ºC -Ti plates were immersed in supersaturated calcium phosphate solutions (Ca/P mol ratio:2.0, Ca2+:3.67 mM, HCO3-:15.09 mM) at 37 ºC for 48 hours to prepare titanium plates coated with an apatite layer (H2O2-Ti-Ap, 300 ºC -Ti-Ap and Ti-Ap). Untreated titanium plates and commercially available cell culture plates were used as controls. Rat MSCs were isolated and primary cultured as described previously. MSCs were seeded on plates at 4.0×104 cells/mL and cultured at 37 ºC in a humidified atmosphere of 95% air and 5% CO2. After 2 hours, the cells attached to each surface were stained using the LIVE/DEAD Viability/Cytotoxicity Assay kit according to the manufacturer's instructions and observed with a fluorescence microscope (Model BX51; Olympus). Living cell adhesion areas were measured using Image-Pro® PLUS software (Media Cybernetics, Inc., Version 7.0). For evaluating osteoblastic differentiation of MSCs, an osteogenic differentiation medium which was minimum essential medium containing 10% fetal bovine serum and 1% antibiotics supplemented with 10 nM dexamethasone, 10 mM β-glycerophosphate and 0.28 mM ascorbic acid-2-phosphate. The cells were seeded on each substrate at 5.0×104cells/mL. After 3 weeks of culture, the osteocalcin contents secreted from the MSCs were measured using a rat osteocalcin ELISA kit according to the manufacturer's instructions. Results and Discussion: Living cell adhesion areas measured after 2 hours culture were as follows: 199±46(300 ºC-Ti-Ap), 220±60 (H2O2-Ti-Ap), 351±96(Ti-Ap), 352±124 (Ti), and 500(TCPs). Osteocalcin contents measured after 3 weeks of culture were as follows: 2987±1237 (300ºC-Ti-Ap), 2430±222 (H2O2-Ti-Ap), 1841±245 (Ti-Ap), 1604±519 (Ti) and 83±133 (TCPs). Cell morphology of rat MSCs strongly correlates with osteoblastic differentiation activity. Correlation coefficient of the living cell adhesion areas and osteocalcin contents of rat MSCs was 0.9589.

Poster Board Number: T-2254

EARLY RESPONSE OF PIG MESENCHYMAL PROGENITOR CELLS TO INTRACELLULAR INCORPORATION OF GADONANOTUBES RESEMBLES ANOIKIS RESISTANCE

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Detachment from the extracellular matrix causes cell death, or anoikis, a process that some neoplastic cells evade during cancer progression to metastasis. We are reporting that mesenchymal progenitor cells (MPCs) respond to the incorporation of gadolinium-loaded carbon nanocapsules, or Gadonanotubes (GNTs), by a non-proliferative, non-apoptotic cell cycle arrest mechanism similar to anoikis resistance, also termed synoikis. In previous studies, we have demonstrated that GNTs can readily labeled MPCs (10^9 Gd^{3+} ions/cell) without significant loss of viability, phenotypic characteristics and differentiation potential. However, GNT-labeled MPCs showed an extended growth lag phase (48 hours) compared to controls (24 hours) and decreased adhesion to collagen and fibronectin. These events were transient, as GNT-labeled MPCs displayed the same properties of control cells after 48 hours. To further characterize the cellular response to GNTs, we investigated possible changes in gene expression by transcriptome analysis. Pig bone marrow-derived MPCs at the log phase were incubated with GNTs for 24 hours. Control and GNT-labeled cells were then re-plated and cultured for another 24 hours, followed by RNA extraction. Microarray (GeneChip Porcine Genome Array; Affymetrix, Santa Clara, CA, USA) data generated from 3 independent experiments were used to compare gene expression changes between control and GNT-labeled cells and to analyze the affected biological pathways (Ingenuity Systems Inc, Redwood City, CA, USA). More than 130 genes were significantly altered, including those involved in cell cycle, DNA damage, and stress response pathways. Data is currently being validated by qPCR, immunocytochemistry, flow cytometry, ELISA, and Western blotting. Flow cytometry studies revealed a significant accumulation of cells at the G2/M phase, indicating that growth arrest is effectively ongoing in GNT-labeled cells. In addition, the expression of DNA replication machinery components, such as MCM2 and 4, CHAF1, CDC45, CKS2, and GINS complex subunit Psf1 were found to be significantly decreased. The G2-M cyclins, B1 and B2, were downregulated, as well as transcription factors and other regulatory proteins that are involved in the control of cell cycle progression and replication, such as E2F1, PCNA, and F-Box protein-5. The cyclin G2, a negative regulator of cell cycle progression, was upregulated. Expression of Angiopoietin-like 4 (ANGPL4) and BNIP3, both implicated in synoikis, were significantly elevated. In order to fully demonstrate that the survival of GNT-labeled cells is assured by a synoikis-like mechanism, we are presently developing oligonucleotide-based assays to knock down the expression of ANGPL4 in GNT-labeled cells. As more investigators, including our group, study the *in vivo* application of biocompatible

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carbon nanostructures as agents for drug delivery, molecular imaging, and cell tracking, we believe that our current findings will be invaluable to the elucidation of the cellular and molecular response to this novel class of nanoparticles.

Poster Board Number: T-2255

THE TRANSPLANTED RAT NEURAL STEM CELLS DOWNREGULATE EXPRESSION OF TLR4 IN CEREBRAL ISCHEMIA OF RATS

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[Objective]Neural stem cells(NSCs) are believed to promote the function recovery in cerebral ischemia in rat. Toll-like receptor TLR4 play a critical role in the neuroinflammatory condition. However, it is not clear that how the transplanted NSCs influence the expression of TLR4 during cerebral ischemia. In this study, we detect the transplanted rat NSCs modulate the expression of TLR4 in cerebral ischemia of rats.[Methods]The NSCs from the embryonic 12.5~14d cerebral cortex of Wistar rats were isolated, identified by the detection of the self-renewal capacity and multi-differentiation potential, labelled through the red fluorescent dye PKH26. The labelled NSCs were transplanted into the cerebral ischemia in rats. The immunohistochemistry and Western Blot technique were performed to discover the expression of TLR4 at several timepoints after NSCs transplantation.[Results]The results of immunohistochemistry suggested that the expression of TLR4 decrease. The Western Blot analysis also revealed that the expression of TLR4 decrease.[Conclusions]These results indicated that the transplanted rat NSCs can downregulate the expression of TLR4 in cerebral ischemia in rats.

Poster Board Number: T-2256

NUNCLONTM SPHERA - A NOVEL SURFACE DESIGNED TO PREVENT CELL ADHESION AND SUPPORT EFFECTIVE GROWTH OF CELL SPHERES IN SUSPENSION

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Stem cells or progenitor cells have the capacity of self-renewal and differentiation at the single-cell level *in vitro*. The formation of spheres in suspension cultures (e.g. neurospheres, embryoid bodies) is widely used for producing different cell lineages from progenitor/embryonic stem cells and is used for further applications. Experimental variability in sphere-forming cultures has been linked to medium composition and volume, cell density, and duration in culture. Nevertheless, the surface property of the culture dish is one of the most critical factors contributing to the success of sphere formation *in vitro*. Although several commercially available culturewares offer properties of low cell adhesion, sphere-forming without random cell attachment and spontaneous cell differentiation is still a challenge to many researchers. The novel Nunclon™ Sphera culture surface by Thermo Fisher Scientific allows cells to grow in suspension with virtually no cell attachment providing superior performance to other commercially available culturewares. In this study, we demonstrate that the new surface supports many different cell types and their ability to generate spheres including: embryonic stem cells, induced pluripotent stem cells, mesenchymal stem cells, HepG2, Hela, HEK293, PC-12, and P19. Over time, the spheres grow in volume indicating that the new surface has no adverse effect on sphere growth. The adsorption of many different substrates, such as Horseradish peroxidase (POD)-IgG, serum protein (human and bovine), Collagen, and Fibronectin, is extremely low suggesting that these large protein molecules do not alter the non-adhesive property of the surface. The improved process of surface modification significantly minimizes batch-to-batch variations, a clear advantage over the existing commercially available sphere culture products. The surface is also animal origin free (AOF) providing benefits during scale-up for clinical purposes. In all, Nunclon™ Sphera offers many benefits over the existing commercially available products and can improve the formation of embryoid bodies and neurospheres aiding in differentiation.

Poster Board Number: T-2257

EFFECT OF A NOVEL SYSTEM OF PORCINE *IN VITRO* MATURATION AND CULTURE ON COLONIZATION OF PUTATIVE PORCINE EMBRYONIC STEM CELLS

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Porcine embryonic stem cells (pESCs) have a great potential in genetic engineering for produce of cloned or gene modified pigs. For derivation of pESCs, the majority source of embryos is *in vivo* produced blastocysts, because they are expected to be high quality. However, obtainment of *in vivo* blastocyst is expensive and toilsome. Thus, many researchers are used blastocysts produced from *in vitro* such as *in vitro* fertilization (IVF) and parthenogenesis (PA). However, it is difficult to establish pESCs from *in vitro* produced blastocysts because in most cases there is no obvious inner cell mass (ICM) or they eventually contain only few ICM cells. Therefore, we investigated the relationship *in vitro* produced blastocyst quality and colonization efficiency of pESCs. In experiment, we performed the IVF and PA with treatment of some chemicals. The control group is produced using M199 media in IVM and porcine zygote medium-3 (PZM3) in IVC. The treatment group is produced using M199 with 2 μM resveratrol (RV) in IVM and PZM5 with 10 ng/mL porcine granulocyte-macrophage colony stimulating factor (pGM-CSF), 2 μM RV and 10 μM β-mercaptoethanol (β-ME) in IVC. Data were analyzed with SPSS 17.0 using Duncan's multiple range test. In total, 1210 embryos in PA, 612 embryos in IVF evaluated. As results, we observed overall blastocyst quality was increased. The blastocyst formation rates were significantly higher ($p < 0.05$) in the treatment groups (54.5%) compared to the control group (43.4%) in PA and hatched blastocysts rates in day 6 and 7 were also increased significantly. Total cell numbers of blastocyst were significantly higher ($p < 0.05$) in the treatment group (55.1) compared to the control group (45.6). In IVF, hatched blastocysts rates in day 7 were increased significantly, too. After seeding porcine blastocyst, the attachment rates were higher in the treatment group (36.2% in IVF and 32.2% in PA) than the control group (26.6% in IVF and 19.5% in PA). Also, colonization rates and cell line derivation rates were higher in treatment group than control group. Colonization rates of control group were 10.8% in IVF and 2.4% in PA, but treatment group were 17.75% in IVF, and 13.1% in PA. The cell line derivation rates were 4.2% (IVF) and 2.4% (PA) in control group. In treatment group, they were 10.0% (IVF) and 7.2% (PA). In conclusion, the treatment of RV during IVM and RV, β-ME , and pGM-CSF during IVC increased quality of porcine blastocysts produced from *in vitro*, subsequently increased derivation rates of porcine putative ESCs.

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Poster Board Number: T-2258

FEASIBILITY OF AN IN-VITRO MODEL -TO EXPLORE THE INTERACTION BETWEEN MESENCHYMAL STEM CELLS AND -CANCER CELLS: USING MYELOMA CELLS AS A TARGET

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Background: Multiple myeloma (MM) is a clonal plasma cell neoplasm characterized by proliferation of neoplastic plasma cells in bone marrow (BM) and osteolytic bone lesions. The MM bone disease is clinically important and is a result of overt activity of osteoclasts and inactivation of osteoblasts (OBs). OBs are normally derived from BM mesenchymal stem cells (MSC) under physiological bone turnover and remodeling. To understand the interaction between the myeloma cells (MCs) and MSC is urgently required; however, which is currently hampered by limited cell numbers and difficulty in detection of the BMMSC in-vivo. Here, a human telomerase reverse transcriptase-immortalized cord blood MSC (hTERT-cbMSC) established from one of our labs (Hung CJ, et al. Cytotherapy, 2010), could be an ideal material for the MSC study. We try to use the hTERT-cbMSC and MM cell lines, to mimic the interaction between the MCs and BMMSC in-vivo, and also to explore the mechanisms involving the inactivation of OBs in MM. Method: Primary BMMSC from MM patients were isolated for in-vitro culture and a co-culture of hTERT-cbMSC and MM cell lines was also used. Multiple phenotypic and genotypic assays on senescence, proliferation, differentiation ability and functional alternations of these MSCs were performed. Results: Primary BMMSC were isolated from newly-diagnosed MM patients (n=9) and non-myeloma control (n=18). The proliferation ability of BMMSC was significantly decreased in the MM patients compared to that in non-myeloma control (0.55 and 0.69, respectively; p=0.047). Similarly, in the co-culture system, while comparing to the control group, the proliferation ability of hTERT-cbMSC was also significantly reduced when it was co-cultured with MM cell lines for one-week (1.125 and 0.947, respectively; p<0.001) and two weeks (1.125 and 0.573, respectively; p<0.001). In addition to the preliminary results from the proliferation assay, the quantification of senescence, differentiation ability and functional alternations of the primary BMMSC and the hTERT-cbMSC from co-culture with MM cell lines will be also presented. Conclusion: The co-culture by using the hTERT-cbMSC and MM cell lines, instead of primary BMMSC and MM cells, may become a suitable in-vitro model for further investigation elucidating the interaction between BMMSC and the MCs in MM.

Poster Board Number: T-2259

NEURONAL NETWORKS FROM STEM CELL DERIVED NEURONS AS *IN VITRO* ALTERNATIVE ASSAY FOR NEUROTOXICITY EVALUATION AND PREDICTION

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Testing of compounds for potential neurotoxic properties is required for all drugs and chemicals in Europe. Due to the complexity of neurotoxic effects especially systemic repeated dose toxicology testing is frequently performed in animals. Here we present a high throughput and high content screening platform based on stem cell derived neuronal networks cultured on microelectrode arrays. These assays allow parallel recording of nine neuronal networks with 28 electrodes each for extended periods of time. thus e can demonstrate repeated dose chronic toxicity as well as acute toxic effects with one integrated assay.

Poster Board Number: T-2260

MONITORING OF THE DAMAGE TO FREEZE-THAWED CELLS BY GLYCAN PROFILING USING A LECTIN MICROARRAY

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Cell-based therapies, such as stem cell transplantation, have become a promising strategy for the treatment of many diseases. New discoveries regarding the regenerative potential of stem or progenitor cells for the treatment and prevention of various diseases have transformed experimental research and led to an explosion in clinical investigations. However, the cell-based therapies have the problems of therapeutic efficiency and safety of cells for transplantation. In addition, cells may be modified or changed after each step of cell processing *in vitro*, i.e. isolation, subculture and freeze-thawing. Therefore, characterization of cell is the most important task for transplantation. Cell surface is covered with various glycoproteins and the cell surface glycans have been assumed to vary among cell types, stages of development and differentiation, and even in malignant transformation processes. In order to analyze the glycan, lectin microarray has been developed. The array is an emerging technology that can be applied to ultrasensitive detection of multiplex lectin-glycan interactions. We reported that lectin microarray analysis was applicable to ES, iPS and other stem cells. In this study, we investigated whether a lectin microarray could be used as a validation tool to monitor cell characteristics after freeze-thawing. Human diploid fibroblast (TIG-1) cells were used in this study as a model system of cellular ageing because they have been well characterized with respect to cellular growth and freeze-thawing. TIG-1 cells at population doubling level (PDL) 24 were assigned to two groups; one was cryopreserved and the other was cultured as control cells (non-frozen). The cryopreserved cells were thawed one week later and re-assigned to two groups; one was cryopreserved and the other was cultivated as the once-frozen thawed cells (Group I). The twice-frozen cells (Group II) were thawed 3 weeks later and subcultivated. We observed cell proliferation and performed lectin microarray analysis of the three groups. In these results, the doubling time of Group II cells was prolonged when compared with that of Control or Group I cells after PDL 50, although the cells of these three groups had similar morphology. However, lectin microarray analysis showed that the signal intensities of Group II cells were significantly higher than those of the other groups at PDL 37 or 38 for Sambucus nigra agglutinin (SNA), Sambucus sieboldiana lectin (SSA), Pokeweed mitogen (PWM) and Urtica dioica agglutinin (UDA). Differences in the cell surface glycan profile after freeze-thawing between Group II cells and the other groups could be detected quickly using the lectin microarray. Our results suggested that a lectin microarray may be an easy and quick method for validation of cellular characteristics. It is hope that cell validation using this system could make it possible to evaluate the condition of various stem cells for transplantation.

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Poster Board Number: T-2261

IMPORTANCE OF PHYSIOLOGICAL OXYGEN AND MEDIA CONDITIONS FOR STEM CELL CULTURE

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Stem cells and differentiated cells intended for research and clinical therapy are almost always grown in incubators flushed with CO2 and air to maintain an aerobic environment and physiological pH in the medium. The oxygen levels under such conditions (atmospheric oxygen; atmosOx) are 20% and are 2-8 times higher than the oxygen levels cells encounter *in vivo* (physiological oxygen; physOx). We, and several others, published a series of studies that showed that culturing differentiated cells at atmospheric oxygen levels significantly skews cellular responses, increases risk of DNA damage and alters the gene expression similar to "stress-response expression". Here we provide evidence to show that physiological oxygen levels are superior for culturing mesenchymal stem cells, iPS cells and other differentiated cells. Using a variety of cellular and biochemical measures of cellular redox status, mitochondrial function, cell surface expression and morphological features, we show that cells cultured at physOx are "healthier", maintain steady growth rate and pluripotency, and are less subjected to culture induced potentially detrimental genomic artifacts than cells cultured at atmosOx.

Poster Board Number: T-2262

PHENOTYPIC MODULATION OF PLURIPOTENT STEM CELLS BY MICROFABRICATION MATERIALS

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Pluripotent stem cells (PSCs; i.e., embryonic and induced pluripotent stem cells (ESCs and iPSCs)) hold a great potential for applications in cell-based therapy, regenerative medicine and drug development/screening due to their unique characteristics, such as unlimited self-renewal without any karyotypical abnormality and differentiation capability into any kind of cells in a body. To control PSC functions, it is necessary to precisely regulate extracellular microenvironments, which have an important role in controlling cellular functions *in vivo* and in vitro. However, it is still a challenge for current PSC experimental settings to study such environments in vitro at a nano/micro-meter scale. Polymer Micro Electro Mechanical Systems (MEMS) technology using a photoresist as a constructional material has recently afforded a simple three-dimensional (3D) fabrication of microfluidic devices, and is advantageous for creating new cellular microenvironmental cues to control stem cell functions due to its great controllability of soluble and insoluble factors on a nano/micro-meter scale. Even though nano/micro-fabrication and mechanical properties of photoresists have been well-discussed, chemical effects, such as toxicity of antimony salt (an optical polymerization initiator) in photoresists and its derivatives, on cell functions are still unknown. Therefore, as the first step of applying MEMS technology in stem cell research, we investigated the effects of MEMS materials on PSC phenotypes and functions, such as pluripotent status, proliferation, cytotoxicity and cellular heterogeneity. First, two photoresist substrates (i.e., TMMRTM S2000 (antimony) and N-S3000 (non-antimony)) prepared in a way typical for photoresist processing were employed for this study. Photoresist substrates treated with gelatin, Matrigel or Corona treatment were tested for their usability for culturing mouse ES cells (mESCs). These results indicated that, while mESCs didn't adhere on the non-coated S2000 and N-S3000 substrates, Matrigel coating provides better mESC adhesion and maintains high expression levels of alkaline phosphatase, which is one of the pluripotent markers. It should be noted that corona treatment and gelatin coating, which are typical approaches for general cell lines, were not sufficient to support mESC adhesion. Second, the capability of photoresists for culturing hPSCs was evaluated. hPSC colonies were cultured on both photoresist substrates. We observed that hPSCs cultured on Matrigel-coated photoresist surfaces in a mTeSR-1 defined medium were able to form colonies over the observation period of 7 days. Next, alkaline phosphatase staining was performed to evaluate their pluripotent status. hPSCs cultured on both substrates highly expressed alkaline phosphatase. Then, a flow cytometric analysis for quantitative monitoring of SSEA4 pluripotent marker expression in individual hPSCs was conducted to investigate how cellular heterogeneity was influenced by these substrates. hPSCs on both substrates showed similar heterogeneity compared with a conventional plastic substrate. These results indicate: (1) photoresists in MEMS are usable for culturing PSCs without feeder cells, and (2) the small effects of antimony salt observed during the 7-day culture, a time span sufficient for maintaining PSCs routinely, encourage further investigations on nano/micro-structured photoresist components.

Poster Board Number: T-2263

SUSTAINED LEVELS OF FGF2 REDUCE DIFFERENTIATION OF STEM CELL CULTURES WITH LESS FREQUENT MEDIA CHANGES

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Cell culture techniques aimed at maintaining a number of different stem cell types in an undifferentiated state involve the frequent addition of FGF2 to the culture media. For human pluripotent stem cells, this is done daily, and for tissue specific stem cells, a typical feeding regime is every 3rd day. Yet even with these laborious feeding schedules, stem cells spontaneously differentiate, indicating that the self-renewal is sub-optimal. We assessed the levels of FGF2 in culture medium with or without added heparin, and found that it is highly unstable, with a 60% reduction after 5 hours and 95% by 24 hours. Hence we postulated that rapid loss of FGF2 could be responsible for stem cell culture differentiation. To address this, we created a cell culture environment with significantly more stable levels of FGF2. We show this new culture method results in higher expression of stem cell markers and decreased differentiation during the expansion phase. Moreover, we reduced the feeding schedule for hESCs to once per 3 days, resulting in significant savings in medium costs and fewer interventions in the cultures, which reduce the risk of contamination. The resulting hESCs were able to differentiate into the three germ lineages as well as cells growth with conventional daily feeding. This new method can be applied to other types of stem cells that respond to FGF2, such as iPSCs or MSCs. We suggest that stabilizing the FGF2 environment mimics stem cell niches that promote maintenance of the stem cell state.

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Poster Board Number: T-2264

SYNTHETIC PEPTIDES AND POLYETHYLENE GLYCOL SURFACES FOR MICROPATTERNING OF PLURIPOTENT STEM CELLS

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Techniques for pattering of pluripotent stem cells (PSCs) are important for a wide range of fundamental and clinical applications, such as studies on stem cell biology, tissue engineering and drug screening using PSC-derived cells. To use surfaces coated with synthetic peptides derived from active domains of extra-cellular matrix (ECM) protein is one of the promising ways to make xeno-free and scalable cell adhesive surfaces as an alternative to conventional surfaces coated with ECM extracted from animals (Z. Melkoumian et al., Nat. Biotech., 2010). In this paper, we report a simple surface modification method for micropatterning of mouse PSCs using synthetic peptides immobilized on a surface via an amine-to-sulfhydryl crosslinkers having poly(ethylene glycol) (PEG) units as spacer (SM(PEG)12, Thermo Scientific Pierce Protein Research Products). Cell adhesive surface is defined through the patterning of synthetic peptide by means of microcontact printing or microfluidic pattering to provide synthetic peptides to the surface previously coated with the crosslinker to bond them through the reaction of the amino group of the N-terminal of peptide with the N-hydroxysuccinimide ester (NHS) group of the crosslinker. The surface without peptide doesn't have cell adhesiveness due to the PEG units in the crosslinker. Microstructures for microcontact printing or microchannel networks were fabricated onto microchips made of poly(dimethylsiloxane) through conventional soft lithography process (G. M. Whiteside et al., Annu. Rev. Biomed. Eng., 2001). The feasibility of selective cell adhesion of peptide-patterned surfaces for culturing mouse PSCs was evaluated by using a mouse induced pluripotent stem (iPS) cell line (mouse iPS-MEF-Ng-20D-17 cell line). Since the crosslinker intrinsically has PEG units to avoid cell adhesion, the present method is more rapid and easy way to make patterned selective adhesion surface without any extra-process for making anti-cell adhesive surfaces.

Poster Board Number: T-2265

LARGE SCALE MANUFACTURE OF CUSTOM TAL EFFECTOR NUCLEASES FOR GENOMIC EDITING.

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Specific DNA-binding transcription activator-like effector nucleases (TALENS) are powerful tools for genome engineering with broad applications in a variety of cell types, including stem cells. The TAL binding domain, from Xanthamonas sp., governs the simple code used to assemble the DNA-binding specificity. We have developed large scale manufacture capability to custom-design TAL nuclease Gateway expression vectors that provide gene editing at targeted loci. In association with GENEART, a TALE monomer library has been created to generate rapid assembly of custom TALE proteins. We are working to optimize the TALE nuclease tool by comparing its activity in a variety of single expression vectors and developing a cell-free QC assay to measure cleavage and repair of specific loci. The TALEN expression clones are being assessed in mammalian cell lines and cell based models. It is expected that TAL effector nucleases will be used in precise genome engineering technologies such as generating IPSC clones, stably modified human embryonic stem cell clones and broader areas of regenerative medicine, research in disease models and the production of therapeutics.

Poster Board Number: T-2266

GENE ACTIVATION USING TRANSCRIPTION ACTIVATOR LIKE EFFECTOR PROTEINS

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Transcription Activator-Like Effectors (TALEs) are proteins secreted by *Xanthomonas Spp* into plant cells to facilitate bacterial invasion. They accomplish this by binding specific promoter sequences and activating the expression of various signaling pathway genes. These proteins are comprised of constant N and C termini flanking repeat domains of various numbers. In nature, there are Tals with commonly between 12 to 18 repeats each repeat consisting of approximately 34 amino acids. These domains are essentially identical in sequence except for 2 central codons' termed repeat variable domains (RVDs). The variation in the RVD sequence determines DNA base binding specificity of each repeat and the combination of different RVD containing repeats determines the sequence specificity of the TALE. Since the TALE 'code' has been solved recently, it has been shown that by engineering RVDs designer TALEs can be made that bind to essentially any sequence in the genome. Here we describe a set of experiments to examine the size (repeat length) requirement for optimal activity of designer TAL activators in mammalian cell model systems. We assay TAL activators with binding target site lengths of 11, 14, 19, 25 base pairs to determine the optimal functionality in our system. Next we use these configurations to attempt activation of endogenous genes by scanning promoter regions with various TALE activators. Ideally, these types of tools could be used in somatic cells to induce pluripotency or in ESC and iPSC to transiently activate specific signaling pathways to direct differentiation toward desired lineages.

Poster Board Number: T-2267

A NETWORK BIOLOGY PLATFORM TO EVALUATE ENGINEERED MAMMALIAN CELLS

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Directing state transitions of mammalian cells in vitro, either through directed differentiation or reprogramming, is a novel paradigm that has transformed our capacity to study otherwise inaccessible cell types in development and disease. While engineered cells are often assayed functionally, a more comprehensive and statistically rigorous approach is needed to define the resemblance of the derived cells to *in vivo* cell types. To address this issue, we developed a computational platform, ClassNet, which uses reconstructed context-dependent transcriptional regulatory networks underlying 44 distinct cell types and tissues to both classify derived cells and ascertain the critical regulatory nodes at which derived cells differ from their *in vivo* counterparts. By applying the platform to all available direct fate conversions of murine cells, we determine the extent to which target cell types have been achieved, identify the cascade of regulatory events that characterize distinct examples of reprogramming, and find unintended, yet predictable, targets of reprogramming factors.

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Poster Board Number: T-2268

CHEMICAL OPTIMIZATION OF BOTH CULTURE SURFACES AND MEDIA MARKEDLY ENHANCES MSC PROLIFERATION

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Culture media contain serum of inconsistent compositions, and plastic tissue culture dishes are inhomogenously ionized due to plasma irradiation. So in conventional cultures, adult stem cells easily lose self-renewal activity and multipotency, and quality of transplantable cells varies depending on culture periods, serum batches and lots of dishes. Recently we developed serum-free chemically defined media STK1 and STK2 for human mesenchymal stem cells (MSC), which are now available from DS Pharm Biomedical, Osaka, Japan. In primay and passage cultures, STK1 and STK2, respectively, enhance MSC proliferation and maintain their multipotency at much higer levels than does DMEM supplemented with 10% FBS. In this study, we developed a new culture system consisting of the serum-free media and self assembled monolayers (SAMs) expressing various functional groups, since conventional dishes were not optimimal for MSC under serum-free conditions. SAMs made of alkanethiols with a long methylene chain give well-defined model surfaces for studies on interfacial phenomena and intermolecular interactions. Although numerous serum proteins easily bind to SAM surfaces, serum-free media allow us to examine direct interactions between cell surfaces and culture surfaces. We made mixed SAMs with various ratios of amino, hydroxyl, carboxyl, and methyl groups. SAMs enhanced proliferation of MSC derived from human bone marrow, synovium and dental pulp in STK2 at a certain ratio of functional groups. The combination of the SAMs and STK1 enhanced MSC proliferation 10-fold in primary cultures compared to plastic dishes with STK1. The combination of the SAMs and STK2 also enhanced MSC proliferation in passage cultures 2- and 10-fold compared to plastic dishes with STK2 or 10% FBS, respectively, within 4-5 days. Some SAMs also enhanced MSC proliferation in the presence of 10% FBS at different ratios of functional groups, probably because serum proteins adhered to these SAMs enhanced MSC proliferation. Furthermore, the optimal ratios of functional groups on SAMs differed among cultures of MSC, fibroblasts and osteoblasts, indicating that certain SMAs can selectively enhance MSC proliferation under serum-free conditions. So this new culture system allowed us for the first time to optimize a whole environment of MSC cultures under chemically defined conditions, and is promising in both basic cell biology and clinical cell therapy.

Poster Board Number: T-2269

SINGLE NUCLEUS RNA-SEQ

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Until recently, single cell gene expression could only be evaluated by conventional methods such as reverse transcriptase-PCR (RT-PCR), which is limited to measuring gene expression from a small number of candidate genes, or microarray analysis that suffers from false positive signals, low dynamic range and the inability to discover new transcripts and novel splice isoforms. With improvements to cDNA synthesis from single cells and the development of next generation transcriptome sequencing methods (RNA-Seq) it is possible to sequence cDNA libraries derived from the < 1 picogram of mRNA from a single cell. This has exposed an unprecedented panorama on the regulation of gene expression at the most fundamental level of the single cell. However, in cases where single intact cells are impossible to recover an approach using a single nucleus is attractive. Extending these methods, we have measured and digitally recorded the steady state mRNA signature of a single nucleus from a mouse neural progenitor cell (NPC) line. Although nuclear transcriptomes were found to be largely similar to whole cell controls, notable exceptions were identified. We also observed a strong averaging effect of the pooled controls from 10 and 100 cells or nuclei and a marked decrease in statistical variability between 1 and 10 cells or nuclei. This underscores the importance for single cell or nucleus resolution - as opposed to bulk sample- approaches for defining cellular uniqueness. These advances will enable gene and transcript expression discovery from tissues where whole cells are difficult or impossible to recover.

Poster Board Number: T-2270

GENERATING ARTICULATED EMBRYOID BODY FOR SPATIALLY CONTROLLED DIFFERENTIATION

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Pluripotent stem cells (PSCs) have the potential to indefinitely proliferate and to differentiate into derivatives from three germ layers. And it was shown that the process for generating embryoid body (EB) could be an initialization step of differentiation procedures. Moreover it was shown by previous research that there were several similarities between the procedure of PSCs differentiation and *in vivo* phenomena. So it is important to develop tools for controlled differentiation of EB spatiotemporally, especially for the field of developmental biology. Here, we show a culture technique to realize exposing EB to spatially different differentiation factors in a microfluidic device. The device consists of upper and lower channels separated from each other by a membrane having a through-hole of 100μm in diameter. All the structures are made of polydimethylsiloxane. First an EB derived from mouse embryonic stem cells (T alpha-1 tubulin-GFP mES cell line. T alpha-1 tubulin is one of markers of early neural differentiation and this cell line express GFP in neural differentiated state) is generated in a well-plate. After EB generation at 24 hours of the culture in the well-plate, the EB is transferred into the upper channel. And the EB is moved and fixed by negative pressure applied from the lower channel. And by injecting different media into the channels, the EB can be exposed to each of the media. In the experiment, we filled the upper and lower channels with FBS 20% medium and neural differentiation medium (ND medium, RHB-A medium, StemCells Inc.) each. And we confirmed that when we actually cultured an EB in the device, the EB became an articulated shape (art-EB), which is like having two spheroids below and above the membrane. On the day 4, the EB was collected from the device and transferred to a collagen-coated dish filled with FBS 20 % medium and the articulated shape could clearly be observed. The extended cells show neural-like shapes in the part exposed to ND medium. Moreover, fluorescence intensity of GFP, which is associated with the expression of T alpha-1 tubulin, was much higher in the neural differentiation part than in the part exposed to FBS 20% medium. This result shows

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that neural differentiation was induced in one spherical part of the art-EB. Another spherical part shows clearly different morphology from neuronal cells and doesn't express GFP. Here, we developed a microfluidic culture technique to form the art-EB, which can be used for spatially controlled differentiation of EB. And it was shown that neural differentiation in one sphere of the art-EB could successfully be conducted.

Poster Board Number: T-2271

BIOLUMINESCENT SYSTEM FOR DYNAMIC IMAGING OF CELL AND ANIMAL BEHAVIOR

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The bioluminescent light technique has been broadly applied to the analysis of promoter activity in cells and the observation of internal structures in the animal body. However, because of their relatively weak luminescence, bioluminescent proteins have been difficult to use for the optical imaging of cultured living cells or in freely moving animals. The advantages of bioluminescence over fluorescence imaging include a low background signal, the ability to observe luminescence without an excitatory stimulus (which can damage cells), and the concomitant preservation of delicate subcellular organelles and structures which can be destroyed by excitation light. Because only living cells that produce ATP support bioluminescent reactions, bioluminescent proteins are useful for identifying living cells, such as in transplantation experiments. This study describes a novel imaging method for single cells and whole mice, by using a new luminescent protein, ffLuc, comprised of a modified form of Venus fused with luciferase. This new luminescent protein enabled the visualization of extending neuronal growth cones and small protrusions on microglial cells in culture. Expression of ffLuc in transgenic mice allowed the fast time-lapse bioluminescent imaging of freely moving mice in hurriedly with a short exposure time. Oral administration of luciferase-containing agarose or topical application of luciferase-containing Vaseline permitted imaging of the stomach and skin, respectively, of the moving mouse. This new luminescent protein can be used to visualize cell migration, differentiation, and division events in cultured cells, and to clarify metabolic disorders, eating behaviors, and drug infiltration in freely moving, nonanesthetized animals.

Poster Board Number: T-2272

ESTABLISHMENT OF AN ALGORITHM FOR AUTOMATED DETECTION OF IPS/ NON-IPS CELLS UNDER A CULTURE CONDITION BY NONINVASIVE IMAGE ANALYSIS

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It is expected for iPS cells as tools for drug development and as resources for regenerative medicine. However, such medical applications demand a stable supply of iPS cells in large quantities. Issues to be resolved include clarification of the mechanism by which differentiated cells become initiated as undifferentiated cells, optimization of the iPS cell generation method, and establishment of a safety assessment method for visual inspection of the cell colony formation process. In this experiment, a cell culture observation system (BioStation CT) was used to observe a culture, and an image analysis software (CL-Quant) was used to scan phase-contrast images and automatically detect iPS / non-iPS cells generated by reprogramming of normal somatic cells. In implementing the automatic detection, an algorithm was designed to categorize cells of different origin based on parameters pertaining to morphological characteristics of the cells. For samples consisting of various numbers of colonies, the coefficient of correlation was extremely high between the number of colonies counted by inspection and the number of colonies counted by CL-Quant. Accuracy in iPS / non-iPS identification was next compared. For the cell type used for algorithm design, accuracy was high at an average of 80.00%. For five types of cells of different origin, the average accuracy was 82.29%. This indicates the high robustness of the method in providing accurate iPS/non-iPS identification results. To confirm consistency of the analysis results, accuracy was compared with the results of analyzing cells stained with the TRA1-60 dye (marker specific to iPS cells) and the AP dye. This experiment shows that iPS/non-iPS identification during the establishment stage can be automated, eliminating the need for human intervention, and allowing for non-invasive analysis without the use of fluorescent dyes. This provides several advantages. 1) Reduction of the required time and effort. The iPS cell identification process previously required several hours. The time and effort used can be reduced significantly by automating this process. 2) Non-invasive analysis. Since the process does not damage the cells, the cells can be reused for the subsequent processes. 3) Homogenization. The method allows for consistent selection of good iPS cells without requiring training. 4) Historical management. By maintaining a record of the identification results, quality evaluations can be reanalyzed with ease. It is expected that by applying this method to time-lapse images and obtaining information on time-variable morphological changes in the iPS cells, changes in intra-cell signals affecting the establishment efficiency of iPS cell, sequential gene expressions after the introduction of the reprogramming factors, and other differences in cloned iPS cells can be observed. The method is also considered to become an effective support tool for establishing regenerative medicine.

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Poster Board Number: T-2273

FERROMAGNETIC NANOPARTICLES FOR MR IMAGING AND MAGNETIC FLUID HYPERTHERMIA

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Introduction: Nanoparticles may serve for both diagnostic and therapeutic purposes. Superparamagnetic or ferromagnetic nanoparticles represent a suitable contrast agent for magnetic resonance imaging (MRI) *in vivo* and for monitoring transplanted nanoparticle-labeled cells by MRI. Ferromagnetic particles can also be used for guided temperature-controlled thermoablation, with labeled stem cells used as carriers. The aim of our study was to prepare perovskite ferromagnetic particles, optimize their size and encapsulation with respect to their magnetic and biological properties, and test them on rat mesenchymal stem cells and *in vivo* as a contrast agent for MRI. Materials: Six samples of manganese perovskite nanoparticles (La1-xSrxMnO3), varying in size over a range of 18 - 21 nm and with different La/Sr ratios, were synthesized. The particles were coated by Si02 to minimize their toxicity and adverse effects on cells; the coating thickness was 20 nm. The particles were added to culture medium with rat mesenchymal stem cells and incubated for 48 hours, then the nanoparticles were washed out and cell viability was evaluated using trypan blue. Cell proliferation was evaluated using the Xcellingence System. Labeled and unlabeled cells and cells mixed with a nanoparticle suspension were exposed to a high frequency magnetic field (1 MHz, 7 mT) to test the influence of the local hyperthermia induced by the nanoparticles. Contrast enhancement in MR images was tested *in vivo* by injecting the nanoparticles into the cortex of a rat and scanning with a Bruker Biospec imager 4.7 T using a T2-weighted image sequence, 1 and 24 days post-injection. Results: The viability of cells incubated with the nanoparticles was in the range of 72 - 85%, whereas a control sample reached 92%. Although the coating substantially improved cell viability, the nanoparticles were still slightly toxic. This also resulted in the lower adhesion of a small part of the labeled cells after 48 hours of incubation. Proliferation was slowed down at the time the nanoparticles were added to the media; however, overall proliferation was not changed. The viability of labeled cells exposed to a high frequency magnetic field decreased by 50% compared to unlabeled ones, although the sample temperature did not increase. This implies that the nanoparticles only heated their immediate vicinity in the cytoplasm sufficiently to kill the cells containing the nanoparticles. In contrast, the viability of cells mixed with a nanoparticle suspension decreased to 15-20% after their exposure to a high magnetic field, along with a temperature increase to 43°C, thus producing an effective termoablation method. The injection of the nanoparticles into the cortex of a rat provided superior MR contrast. Conclusion: Coated perovskite nanoparticles are only slightly toxic and may be improved by further surface functionalization. After their injection, the nanoparticles can be easily tracked by MRI *in vivo*. Due to their ferromagnetic properties, they can be locally heated by an external high frequency magnetic field. Therefore, the nanoparticles can be used for image-guided thermoablation with stem cells used as carriers.

Poster Board Number: T-2274

STEM CELL-DERIVED MOTOR NEURONS AS AN ATTRACTIVE APPROACH FOR HIGH-THROUGHPUT SCREENING OF BOTULINUM NEUROTOXIN INHIBITORS

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Clostridium Botulinum is a soil dwelling, gram-positive bacterium that produces botulinum neurotoxins (BoNTs), one of the most potent groups of neurotoxins known. BoNTs specifically target motor neurons, where upon entering the cell via receptor-mediated endocytosis, particular subunits of the SNARE complex are cleaved by the BoNT light chain metalloprotease, preventing the release of vesicles containing neurotransmitter acetylcholine, resulting in flaccid paralysis. Due to these toxins' rapid mechanism, as well as the ease of their production, they are considered potential bioterrorist weapons. Traditional approaches to screen for small molecule BoNT inhibitors have used enzyme-based in vitro assays, however the lead compounds often fail in subsequent cellular evaluations due to poor uptake and/or compound instability. Establishing a cell-based, high-throughput screening model can reduce these false-positive results. In order to develop this system of identification, a large, relatively pure culture of motor neurons is required. Traditionally, immortalized neuroblastoma have been used in BoNT cellular studies, however they are insensitive to BoNT, requiring extreme doses to achieve sufficient intoxication. Thus in a high throughput screening campaign that most accurately mimics the *in vivo* condition, these cells would be disfavored relative to primary motor neurons. However, it is impossible to obtain a sufficient number of motor neurons for screening purposes using standard harvesting techniques (e.g. primary mouse spinal cord). The differentiation of stem cells into motor neurons would provide an alternative method for producing the purity and quantity of cells needed for potential inhibitor assessment. By exploiting the capacity of stem cells for constant proliferation, a cellular-based screening of BoNT inhibitors is made possible. The protocol of differentiation used in this presentation involved four distinct stages and the use of small molecules. Transitions were confirmed by immunocytochemistry, and later stage cells were positive for neural markers Olig2 and β-tubulin (Tuj1). The derived motor neurons were evaluated by intoxication with purified BoNT/A, the cleavage of SNARE proteins was analyzed by western blot, and the results compared with the other cellular and *in vivo* models of BoNT/A intoxication.

Poster Board Number: T-2275

COMBINATORIAL APPLICATION OF MULTIPLE MICRORNAS TO INDUCE NON-INTEGRATIVE CELL TRANS-DIFFERENTIATION ON MICROFLUIDIC CHIPS

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A fundamental challenge for stem cell technology is finding the right culture conditions for cell expansion, differentiation and reprogramming. Each process involves multiple genes, extracellular factors, and intracellular signaling pathways. With currently available tools, however, multifactor experiments are labor intensive and difficult to carry out reproducibly. Microfluidic technologies, coupled with biocompatible materials, employ precise control of microenvironment of cells, facilitate studies of multi-factorial com-

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binations, and enable development of robust, reproducible and chemically defined cell culture systems. Using Multilayer Soft Lithography, we have developed a microfluidic chip and an automated instrument that can culture cells on chip for extended period of time and deliver multiple combinations of different factors to cells. Each chip includes thirty-two cell culture microchambers and eight reagent inlets. Reagents can be automatically multiplexed to desired combinations and ratios at various pre-defined time points. Cells can also be harvested from the chip for continued off-chip culturing, single-cell genomic analysis, and/or functional assays. We demonstrate on-chip trans-differentiation of human BJ fibroblasts to neurons by direct transfection with combinations of synthetic microRNA mimics. The identities of cells were confirmed with immunostaining and gene expression profiling. This non-integrative approach of miRNA administration resulted in trans-differentiation with high efficiency and cell viability. In summary, the microfluidic system reported here provides the potential to study and screen the precise combinatorial effects of multiple factors on cell culture maintenance, reprogramming and differentiation, hence could be a valuable tool for the stem cell research community.

Poster Board Number: T-2276

STREAMLINED ANALYSIS OF HETEROGENEITY IN STEM CELL POPULATIONS USING SINGLE-CELL GENE EXPRESSION PROFILING

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Single-cell gene expression profiling has recently been used to characterize emergent properties in cell populations that drive lineage choice and specificity in reprogrammed cells; resolution of cell fate decisions in very early embryonic development, and for identification of cancer stem cell biomarkers in tumor biopsies. We have developed a simple, modular workflow for streamlined analysis of cell populations down to the single-cell level. The workflow is centered on two key components: a new benchtop system for automated cell isolation and cDNA preparation, and the Biomark HD™ system for highly parallel gene expression analysis. Starting from samples containing only a few hundred cells, the preparation system isolates individual cells into discrete compartments within a microfluidic device. Cells can be inspected after isolation using either brightfield microscopy or fluorescent markers to verify cell number and type. Following cell inspection and verification, the selected individual cells are automatically processed to prepare and output high-quality targeted cDNA for gene expression analysis. The targeted cDNA samples are then loaded and analyzed with up to 96 gene expression assays in parallel on Dynamic Array™ IFCs using the Biomark HD system. This workflow has been used to identify and analyze underlying heterogeneity in a variety of cell types and provides a general method for detailed analysis of stem cell cultures.

Poster Board Number: T-2277

GENERATION OF MATURE DOPAMINE NEURONS BY MANIPULATING EXOGENOUS NURR1 EXPRESSION PATTERN REFLECTING THAT IN THE DEVELOPING BRAIN

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Neural stem/precursor cell (NSCs/NPCs) cultures can be a source of dopamine (DA) neurons for experimental and transplantable purposes. Nurr1, a midbrain developmental gene, is regarded as a potent factor to overcome limited DA neuron differentiation from cultured NPCs. However, forced expression of Nurr1 in NPC cultures generates non-neuronal/immature DA cells. We show here that the level and period of Nurr1 expression critically affects neuronal differentiation and maturation of Nurr1-induced DA cells. Mature DA neurons were generated by manipulating the Nurr1 expression patterns into patterns similar those in the developing midbrain. This research was supported by a grant KRF-2011-0008952 funded by the Ministry of Education, Science and Technology, Republic of Korea.

Education and Outreach

Poster Board Number: T-2278

REPROGRAMMING STEM CELL UNDERGRADUATE EDUCATION

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The emerging discipline of stem cell biology is a scientific field uniquely affected by the political environment and ethical standards. Stem cell biology has radically transformed how we think about cells, development and disease, and is equally transforming the educational priorities of our undergraduate and graduate teaching programs in the biological sciences. Topical texts and undergraduate teaching materials on stem cell biology are scarce or non-existent, in part because the science itself progresses so rapidly. In addition to these challenges, human stem cell biology offers unique, cross-disciplinary learning opportunities in biomedical ethics, law, and public policy. Finally, traditional and didactic methods of science instruction often fall short of producing enduring ways of learning, especially in rapidly changing disciplines of the life sciences. In the fall of 2010, a team of educators and scientists at Stanford, Harvard and local community colleges galvanized by their common interests in science education came together to address these needs. The group began a three-year project to develop the nation's first formal, multidisciplinary, undergraduate curriculum in stem cell biology targeted at students in technical training programs that fuel the life sciences workforce. Resolving to "teach more by lecturing less" the team employed backwards design methodologies to produce the first of two suites of teaching and learning materials, titled From Embryo to Beating Heart. This paper describes the first eighteen months of our effort to devise a novel pedagogical approach for teaching stem cell research. The content development work resulted in 54 hours of instructional materials in stem cell research for undergraduates preparing for careers in the life sciences. Elements include 1) in ten topical areas, a set of contextually-driven lectures and accompanying 5-10 page introductory reading assignments; 2) complimentary laboratory protocols for the derivation, maintenance, and differentiation of mouse and human embryonic stem cell lines, including directed

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differentiation of neural and cardiac cell types; 3) learning assessments with pre-and post-tests, embedded, open-ended questions and case studies for group discussion, quizzes, laboratory notebook rubrics, and a multi-week concept mapping exercise; and 4) two major activities for student pairs designed to connect themes and disciplines. The first activity features a set of mini-experiments focused on directed differentiation of embryonic stem cells, the second engages students in a public policy debate on the regulation, law, and ethics of stem cell research. We describe the methodology used to design of the course, and present preliminary data on one of our evaluations, the concept mapping exercise. We then discuss the challenges and successes of our Spring, 2011 launch of the course at Stanford and other sites, paying particular attention to whether the students were able to successfully integrate major concepts into their understanding of stem cell research.

Poster Board Number: T-2279

MODIFIED TECHNIQUE FOR EXTRACTION OF DENTAL PULP STEM CELL

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ABSTRACT Various techniques have been reported in the literature for the isolation of Dental pulp stem cells (DPSCs), those techniques were summarized by Huang et. al 2006 as: 1-The outgrowth method (Tsukamoto et. al 1992) 2- The enzymatic digestion using collagenase/dispase/trypsin EDTA (Onishi et. al 1999, Gronthos et. al 2000) Since the most widely used technique was the one proposed by Gronthos et. al 2000, The Purpose of this study is to test the validity of a MODIFIED technique based on Gronthos et. al technique, used for extraction of human dental pulp stem cells. Material and methods Dental pulp stem cells were isolated from human pulp tissue of extracted teeth after patients' signed a consent form. The pulp content of every 2 teeth were mixed and subjected to enzymatic digestion with Collagenase type I (1mg/ml) and dispase (5,000 Ca seiniytic units). Cells were then cultured on different plates using cell culture medium. The morphology of the resultant cell lines was evaluated microscopically through hematoxylin and eosin staining (H&E), as well as for their colony forming ability to confirm the presence of stem cell morphological features. Additionally for characterization of these extracted dental pulp stem cells a Fluorescence-Activated Cell Sorting (FACS) analysis, and Immunocytochemical staining was utilized to evaluate the expression of Vimentin in cellular cytoskeleton. Osteogenic and Adipogenic differentiation was induced and evaluated through cytochemical staining, in addition Real time Polymerase Chain Reaction was used to measure certain osteogenic and adipogenic gene expression in different time points. Results: After 5-10 days in culture, few scattered spindle shaped cells were present, attached to the plastic surface of the culture plate. H&E staining illustrated cells with predominant spindle-shaped appearance and different sizes. The nucleus was central, large, pale with 2 or more nuclei in some cells and prominent multiple nucleoli. In 7 days, cells from the second passage formed several colonies, with more than 50 cells in each colony, as evaluated by crystal violet staining. Images for cells in the fourth passage demonstrated samples positive to Vimentin marker. FACS analysis revealed a high expression of stromal cells-associated markers CD105,CD90, CD73 as well as CD13 ,CD29 CD44 throughout the samples (>95%) while they were negative for hematopoietic markers CD34, CD45, CD14, CD31, as well as for the MHC class II HLA- DR molecule. Osteogenic induction resulted in marked expression of alkaline phosphatase, osteopontin as well as osteocalcin markers as evaluated by RT-PCR, with the highest fold induction at day 14, and induced cells were positive to ALP staining as early as day 3, however, only little adipogenic differentiation was evident both by RT-PCR evaluation for Adiponectin, PPAR-[[Unsupported Character - ɤ]] and Activated Protein-2 marker and with Oil Red O stain for similar time periods. Conclusion: The modified technique presented here simplify the procedure for human dental pulp cell isolation by a) Defining the number and the source of teeth, b) reducing the concentration of digesting materials and c) reducing the time of the procedure. The cells isolated by this technique shows mesenchymal cell potentiality and could be utilized for future studies concerning dental pulp stem cell. This modified technique could be utilized for future studies concerning dental pulp stem cell extraction

Ethics and Public Policy

Poster Board Number: T-2280

INTERNATIONAL HESC REPOSITORIES

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It is well established that scientific advances are dependent on collaborative, international research--as science is not constrained by geography. As a result, researchers, universities, private enterprises, and other interested parties are increasingly facing uncertainty related to the diversity of regulatory schemes that govern human embryonic stem cell ("hESC") research at local, state, national and international levels. Nonetheless, even in this uncertain scientific, political and commercial climate, OECD countries, including most of the G8 nations, have committed considerable public investment in hESC research. Public investment in hESC research has increased, in part, because it is a key aspect of life science innovation taking place in what is known as the "knowledge economies" of developed nations. This most recent form of scientific innovation has also quickly turned into a discourse of political controversy around the world. The most debated issues relate to various strategies to obtain hESCs (e.g., left-over embryos from IVF treatment or from embryos created through parthenogenesis or SCNT), in addition to hESCs application in medical research (consent and donor information). While standardization is imperative to science, a less publicized obstacle to advancing hESC research is that the field is poorly standardized compared to more established fields of biomedical research (e.g., genomics). Accordingly, basic aspects of classification, culture protocols and specimen handling protocols are not universally standardized for hESCs, which has hindered scientific collaboration nationally and internationally. The science-minded legal community, in collaboration with interdisciplinary stakeholders, have the ability to facilitate the advancement of transnational hESC repositories through standardization. Consistently structured hESC repositories would ensure that appropriately characterized hESCs function in the intended manner when applied in research or clinical settings. Facilitating the creation of transnational hESC repositories would thus increase the movement towards the standardization of the storage, transfer, and laboratory protocols for handling hESCs. The legal community successfully helped develop a blueprint for basic human tissue repositories and is uniquely positioned to help facilitate the creation of standardized, transnational hESCs repositories, similar to the model UK Stem Cell Bank. And, given that the UK's hESC repository has emerged as a key benchmark towards standardization of protocols on the national and international level, continuing this momentum can help depoliticize some of the issues related to hESC research by

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providing a procedural solution that is less conflict ridden. While the law cannot fully mitigate the uncertainty with the complexity of regulatory schemes based on political regimes that ultimately govern public funding mechanisms related to hESCs, the law can effectively create governance and management infrastructure through hESC repositories to enhance quality for both research and clinical practice. Working with the legal community to develop hESC repositories that create technical standardization criteria and enforce key material standards for hESC lines will mediate relationships between laboratories in the field of hESCs--while maintaining both technical and ethical standards for the benefit of the public.

Poster Board Number: T-2281

EMBRYONIC STEM CELL RESEARCH IN SAUDI ARABIA; THE CURRENT PRACTICE AND FUTURE DIRECTIONS

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Recent advances in the field of cloning and stem cell research has raised many complex questions. It is rare that a field of science causes debate and challenge not only among scientists but also among ethicists, religious scholars, governments and politicians. There is no consensus on the morality of human cloning, even within specific religious traditions. In countries in which religion has a strong influence on political decision making, the moral status of the human embryo is at the center of the debate. We will discuss our experience; how Islamic teachings make this very promising research and therapeutic technique, and modality of treatment permissible; and the Islamic perspectives about stem cell therapy, in particular embryonic stem cell therapy. Embryonic stem cells (ESCs) have huge therapeutic potential because they can give rise to every cell type in the body (pluripotentency) as compared to stem cells from certain adult tissues which can only differentiate into a limited range of cell types. For this reason scientists stress the importance of embryonic stem cell research. However, this research raises sensitive ethical and religious arguments, which are balanced against possible great benefit of such research for the patients suffering from so far incurable diseases. Serious questions remain about safety. In Saudi Arabia for the last five years stem cell research has been established with launching of several projects. One of these projects is embryonic stem cell therapy for Genetic Metabolic disorders which is one of the most promising modalities for the therapy and prevention of mental and physical handicap in children. In conclusion, it is still unclear which human stem cells_whether embryonic or adult_will be developed and for which conditions. Qualities of the ideal stem cell in a clinical setting are expected to be extensive and far reaching. The ability for stem cells to be expanded in culture without genetic and epigenetic abnormalities, their ability to form functional cell types in vitro and *in vivo*, and their immuno-compatibility with the patient need to be studied. Given this, the focus of research community, should be on developing human research capacity in both ASCs and ESCs. Each type of research will take time to mature. The ethical debate will need to produce acceptable policy and regulatory compromises so that the regulatory burden can be reduced and investors' risk aversion can be overcome. If these things happen, the major remaining barrier to realizing the medical benefits of stem-cell research might be the lack of skilled scientists in the field. Our experience in Saudi Arabia will be presented.

Poster Board Number: T-2282

RISK PERCEPTION AND COMMUNICATION IN STEM CELL TRIALS FOR SCI

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In November 2011, the sponsor of the first stem cell trial for spinal cord injuries (SCI) halted its study prematurely due to "capital scarcity and uncertain economic conditions". The abrupt discontinuation of this trial raised immediate questions about the informed consent of participants: whether or not they were informed about the 'risk' of the trial stopping early, whether such risk would be 'acceptable' in first-in-human trial settings, and how participants perceived the materialization of this risk in the light of their decisions to participate. Risk perception and risk communication play a major role in the translation of stem cell research to clinical trials and treatment. These are important elements in the informed consent process and are relevant to an individual's decision to participate in a trial. However, both are fraught with difficulties. FDA guidelines on clinical trials require that informed consent documents disclose all reasonable and foreseeable risks, but we know that such documents are not always effective in actually communicating and informing participants about potential risks [Anderson & Iltis, 2008]. Further, in the stem cell domain, risk communication is a complex matter; many medical risks are still unknown and perceptions of non-medical risk remain underexplored. Our focus is on the following questions: What risks are relevant to prospective trial participants? How do individuals perceive different risks? And, how can risk communication in stem cell research be optimized? We explore these questions toward the development of a theoretical framework for risk communication in first-in-human trials. Building on the existing literature that has largely concentrated on medical risks, we assess the extent to which non-medical risks, including emotional or social risks, the risk of exclusion from future trials, and the early stopping of a trial should be communicated, and explore best practices to address them. Anderson, E. E., & Iltis, A. S. (2008). Assessing and Improving Research Participants' Understanding of Risk: Potential Lessons from the Literature on Physician-Patient Risk Communication. Journal of Empirical Research on Human Research Ethics, 3(3), 27-37.

Poster Board Number: T-2283

A TALE OF TWO EGGS: CALIFORNIA POLICY ON CONTRIBUTING OOCYTES TO STEM CELL RESEARCH AND ITS ORIGINS.

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Like the rest of the United States, California has an active, if niche, human oocyte trading industry which is an offshoot of the private fertility sector. Large-scale financial compensation, generally US $3500 and up, in exchange for oocytes is the norm and other than Federal requirements to report their success rates to the Centres for Disease Control, there are very few regulations surrounding fertility treatment. However, eggs that are destined for stem cell research laboratories must be donated voluntarily with only the most minimal reimbursement for expenses. Depending on the location and funding source of the research, stem cell research protocols involving oocyte contribution may be subject to some combination of two institutional review boards, two sets of national guidelines, one

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set of state regulations and two state laws. In the California context, selling eggs to make babies, it appears, is both ethical, acceptable and none of the State's business. Selling them to make stem cells, however, is ethically indefensible and even exchanging them merits the careful intervention of public regulators. The process by which eggs are extracted from women is identical whatever their ultimate ex-vivo destination: why should it be ethical and acceptable to offer large sums of money for them in one situation and not in another? The situation is further confused by the fact that New York, after careful deliberation, made the opposite policy decision, declaring that oocyte contributors to research should receive equivalent compensation to those who contributed to fertility treatments. A policy that contributed to one of the sole successes in somatic cell nuclear transfer (SCNT) research in recent years. This paper argues that the situation surrounding the contribution of oocytes to stem cell research is both shaped by and reveals larger schisms in political forces surrounding reproduction, research and the delineation between the public and the private in the US. In particular, historical debates between libertarian and communitarian approaches to the regulation of private life have splintered existing policies surrounded assisted reproduction, abortion and gamete donation in ways that appear radically inconsistent. Although the relevant association between these broader issues and SCNT research may not be immediately apparent, these larger forces had a formulative impact on the formulation of oocyte donation policy in California, and by extension other venues. We will explore how these policies developed and their contribution to stem cell policy in California.

Poster Board Number: T-2284

OBTAINING ADULT HUMAN STEM CELLS THROUGH CLAIM OR CONTRACT: A LIBERTARIAN PERSPECTIVE

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In the 1990 case of Moore v. Regents of the University of California, the Supreme Court of California held that adult stem cell lines could be derived (and later patented) from "waste" tissues of a patient without requiring the researchers to first obtain consent. This contentious ruling must be seen in light of previous rulings, laws, and presumed governmental duties. When one assumes only a minimal libertarian understanding of physician/researcher-patient interactions and governmental duties, I argue that the position of the court is flawed on the basis of actual research practices for obtaining the stem cells. For the researcher to claim a right to any tissue removed from a patient (and thus formerly owned by that patient), the tissue must be said to have been abandoned by the patient and thus open to homesteading by the researcher and others; otherwise the researcher will be violating the patient's ownership rights over that property. Yet when holding any reasonable standard for abandonment of property, the tissue removed during surgery or sampling does not qualify as it must be intentionally preserved and modified before a sufficient amount of time has passed for its ownership by the patient to have wither away. Instead of being able to claim stem cells from removed tissue on the basis that that tissue has been discarded and abandoned, the researcher may only acquire these stem cells from the patient via the other means of property acquisition, a contractual arrangement. In such a contractual arrangement, the patient as original and absolute owner retains all rights not explicitly granted to the researchers. Thus, the right to conduct various types of research leading to patenting of stem cells must be granted by the patient for the researcher to possess such rights. The patient may choose to void any claim to his or her former tissue and grant the researchers absolute rights, but given that many governmental protections of modern society are not assumed in a libertarian framework, it is far more likely that the contract will confer only conditional ownership to the researcher, either specifying certain types of research that are acceptable or unacceptable. Finally, the special features of stem cells_theoretically perpetual renewal and identifiability_coupled with the exponential growth of biotechnology in the private and public sectors result in the potential for gross privacy breaches and perhaps unintended harms resulting from future stem cell research, which only fully informed consent about future research can adequately guard against. Inasmuch as the withdrawal of stem cells requires some involvement of a health-care worker and the potential violation of bodily integrity if the tissue removal is unwanted based upon information that could have been provided to the patient, to withhold such information before the procedure would be tantamount to battery. Even a minimal libertarian government has an interest in protecting its constituents against such harms, and thus the licensure of health-care workers will necessarily include the requirement of informed consent before such procedures. The researchers, though not party to the surgery/donation itself, will nevertheless be bound by the conditional ownership granted by the informed consent agreement.

Poster Board Number: T-2285

DATA MINING ON NIH FUNDING DISTRIBUTION TO STEM CELL RESEARCH

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Stem cell research continues to gain momentum in the biomedical research and drug discovery field because of its renewable cell source in nature and potential to enable better treatments of human diseases. This study examines the National Institute of Health (NIH) funding distribution to stem cell research at state level and compare their percentage shares of human non-embryonic stem cell research vs. human embryonic stem cell research from FY2008 to FY2011 (non-ARRA). In 2008 total NIH funding allocated to stem cell research was over $930 million, which accounted for about 4% of the overall NIH spending on extramural funding. By analyzing data on NIH RePORT website, the top five states received stem cell research funding in 2008 were California (CA) at16.3%, Massachusetts (MA) at 10.9%, New York (NY) at 7.6%, Washington (WA) at 5.7%, and Pennsylvania (PA) at 5%. These five states received close to half (46%; $426 million) of all NIH awards devoted to stem cell research. Compared with the funding distribution in 2010, CA (16.6%), MA (11.2%) and NY (9.2%) showed moderate growth on competing stem cell research support whereas the shares of WA (4.8%) and PA (4.7%) have declined slightly. The emerging efforts from state initiatives and strong institutional supports in CA, MA and NY might have leveraged growing shares of federal dollars. Human stem cell research plays a pivotal role on transforming laboratory based research into life saving therapies successfully. Human stem cells and their derivatives are essential assets for toxicity test, drug screening or cell-based transplantation down the road. Further analysis of the funding subcategories indicated that in 2010 NIH spent $467 million to support human stem cell research - 73% to human non-embryonic stem cell research and the remaining 27% to human embryonic stem cell research. CA, MA, NY, WA and PA each shared 13.2%, 10.3%, 6.9%, 7% and 3.8% respectively of total NIH funding in the area of human non-embryonic stem cell research. Human embryonic stem cell research appeared to be more

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prominent in states like CA, MA and WA. CA was the most highly-funded state and accounted for 28.4% of all NIH funding to human embryonic stem cell research compared with MA at 12.1%, NY at 5.1%, WA at 12.6% and PA at 4.1%. Pie charts and bar graphs will be used in the poster to best illustrate the NIH stem cell funding trends and distributions.

Poster Board Number: T-2286

AN ELSI APP FOR STEM CELL SCIENCE AND MEDICINE

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In this study, we explore how Internet cloud computing, driven by apps and social media, can play a role in disseminating to relevant stakeholders information about the ethical, legal, and social implications (ELSI) of stem cell science and its clinical translation into regenerative medicine and cell therapy. This pursuit arises not only against the backdrop of cloud computing, apps, and social media growing generally in all scientific, commercial, industrial fields, but specifically in the life sciences, medicine, and health care. Applications of cloud/apps/social media computing and communications technologies in life science/medicine/health care include: use by patients and consumers for obtaining information about diseases and treatments and communications among those suffering from a common ailment; medical students seeking educational tools that they can view on their smart phones; physicians seeking information about new medicines and procedures and efficient means of communicating with their patients; and various government agencies and policymakers engaged in health care and facing the need to communicate with each other as well as their various constituencies. The format of the digital content in these communications includes text narratives, podcasts, videocasts, and various types of graphics. Beyond use for information dissemination and education, apps and social media networks are being extended to information collection in clinical trials. Moreover, in health care settings, mobile apps have become available for remotely collecting observations of patients' vital signs and wirelessly transmitting these observations to professionals in central locations responsible for monitoring multiple patients simultaneously. These trends present at least two questions in the intersection of ELSI, internet cloud/apps/social media, and life science/medicine/health care: 1) how to assure use of such technology comports with "good" or "best" practices within the tenets of ELSI; and 2) how to use this technology to communicate ELSI-related information to patients, consumers, medical students, physicians, government officials, policy-makers and others in life science/medical/health care centers of activity? We focus on the second of these two questions, particularly as it relates to stem cell science and medicine. We hypothesize that an app can be developed for communicating such information to the various stakeholders. We examine this hypothesis by proposing the design of an app that will be international in scope. Its content will include: various formative documents giving rise to bioethics generally (e.g., the 1979 Belmont Report) and stem cell-related bioethics (e.g., the 1984 Warnock Committee); various documents reflecting the translation of bioethical principles into statutory and regulatory requirements (e.g., the U.S. Federal Policy for the Protection of Human Subjects (the "Common Rule"), the U.K. Human Fertilization and Embryo Act of 1990, and the 2009 NIH Guidelines for Human Stem Cell Research); and the extensive body of relevant writings from bioethicists and legal commentators. We envision that this app would be available on an open source basis.

Poster Board Number: T-2287

THE NIH HUMAN EMBRYONIC STEM CELL ELIGIBILITY WORKING GROUP: THE FIRST TWO YEARS

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In this presentation, two inaugural members of the National Institute of Health's Human Embryonic Stem Cell Eligibility Working Group look back across the last two years, and discuss the issues the Working Group faced and how they have resolved them. In March 2009, United States President Barack Obama fulfilled a campaign promise by expanding the number of human embryonic stem cell (hesc) lines available for research use with public funds. Hesc lines that fulfilled the administration's criteria were considered eligible for funding and were entered into a public registry. In order for hesc lines to be eligible for use in research with public funding, they must have been originally created for reproductive purposes, and donated, without financial incentive, by the individuals who sought reproductive treatment, and who provided written consent for their embryos to be used for research. During the public comment stage on the proposed regulations, many scientists expressed concern that existing stem cell lines, including those approved under the Bush administration, would not be eligible for funding under the Obama rules because of slight deviations from the new requirements, deviations that might not be ethically significant. In response, NIH created a Working Group (WG), whose first task is to review applications for embryonic stem lines created before March 2009. The WG's job is a combination of "history detective" and IRB. It determines whether the lines were derived from embryos originally created for reproductive purposes, and whether the donors gave their embryos for research without financial incentive. It looks backwards to determine the quality of consent given by embryo donors years ago for cell lines being scrutinized today. The WG's second task is to evaluate lines for which embryos were donated in countries other than U.S. Was the consent process adequate? Were the embryos donated without coercion? How does one apply these important concepts across different cultures? How should the WG take into account factors that do not exist in the U.S., such as legally mandated limits on how long human embryos may be frozen? Are these limits coercive in ways that weaken informed, voluntary consent of the donors? The WG makes recommendations to the NIH Advisory Council to the Director, which makes its own recommendation to the Director, who makes the final decision. Since its inception, the WG has recommended approval of 48 lines and rejected 65 lines. The Director has accepted all of the WG's recommendations. Lines were recommended for disapproval based on variety of concerns, including lack of clarity in the consent process, and lack of IRB approval. The WG also engaged with a number of novel issues, often leading to new NIH policies. For example, it was determined that when a consent document informed prospective embryo donors that their embryos would be used for research on a specific disease or disease cluster, the lines would be approved only for research in that area, thus "keeping faith" with the donor.

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ESTABLISHING AN EVIDENCE-BASE AND ASSERTING LEGITIMACY IN THE CLINICAL TRANSLATION OF STEM CELL SCIENCE: AN EXAMINATION OF INTERNATIONAL GUIDELINES FOR THE INNOVATIVE USE OF AUTOLOGOUS ADULT STEM CELLS.

Lysaght, Tamra

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Stem cell science is an emergent field of research that offers enormous potential in the treatment of many significant diseases, illnesses and conditions. However, the clinical translation of stem cells raises many regulatory and ethical issues that yet to be fully explored. One such issue concerns the evidence-base that should be required for the clinical use of novel stem cell based-therapies. Many argue that evidence should be gathered in formal clinical trials to first demonstrate the efficacy and safety of an experimental intervention before it is accepted into clinical practice. However, others claim that novel interventions with adult stem cells for autologous use are innovative medical practices that need not be subject to formal clinical trial. The clinical guidelines of two professional organisations - the International Society for Stem Cell Research and the International Cellular Medicine Society - have each adopted these divergent approaches. In this paper, I examine the guidelines of both organisations to identify the tensions between them regarding the establishment of an evidence-base for the clinical use of autologous adult stem cells. I argue that through these guidelines, both organisations are attempting to establish legitimacy for their version of an evidence-base for stem cell medicine and assert epistemic authority over the clinical translation of stem cell science. These claims to authority are made through proscriptions for the type of evidence that should be required, how it should be validated and who should oversee the collection and validation of evidence before novel stem cell applications are accepted into clinical practice. I conclude by reflecting on some of the implications involved in developing a framework for the oversight of innovative stem cell interventions.

Poster Board Number: T-2289

NEXT GENERATION SEQUENCING OF INDUCED PLURIPOTENT STEM CELL LINES: ETHICAL AND POLICY CONSIDERATIONS.

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Stem-cell genomics promises to improve our ability to prevent and cure disease by providing cells for organ transplantation and cell therapies. It will also enable the creation of successful model systems for drug discovery and contribute to the understanding of the processes of human cell differentiation for the treatment of several diseases including cancer. For instance, the use of next-generation sequencing (NGS) technologies in induced pluripotent stem cell (iPSCs) lines is expected to greatly contribute to the development of new testing methods for drug efficacy, toxicity and safety for individuals, thereby expediting progress towards personalized medicine. The challenges posed by stem-cell 'omics' efforts are not constrained only to scientific hurdles, the field is also confronted by an array of ethical, social and policy concerns as pertains for example, informed consent, feedback of incidental findings, privacy and the governance of research. While, some of these concerns are not completely new, the old answers might not suffice given the scale of challenges. NGS methods are now routinely applied to iPSCs lines, allowing fine, detailed, genotypic information of the cell lines at high resolution. Given that such iPSCs are (often) derived from living individuals - including paediatric populations - are contemporary mechanisms for protecting the autonomy, privacy and confidentiality of donors sufficient? Are current governance procedures adequate? In an era of stem-cell genomics what is the appropriate role for regulatory bodies? This presentation will provide an overview of some key ethical issues surrounding applying next generation sequencing techniques to stem cell research. Issues surrounding informed consent, privacy and confidentiality, data security,feedback of research results and governance of research will be addressed.

History of Stem Cell Research

Poster Board Number: T-2290

TRENDS AND COMPETENCIES OF STEM-CELL RESEARCH: A BIBLIOMETRIC PORTFOLIO ANALYSIS ACROSS USA, UK AND JAPAN

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Stem-cell research has been an active growing scientific field with substantial potential for future pharmaceutical and clinical applications. Scientific investigators in various fields, not only in the biology/medical field but also in interdisciplinary fields such as chemistry, material science and sociology, have been proactively conducting studies aiming to address stem-cell related issues. Similarly, government and non-government organizations have dedicated substantial amount of resources/funds to facilitate both studies and development of related research centers. Despite its key importance to the huge amount of resources/funds spent on the field, there is still little amount of studies dedicated to explore the trends in stem-cell research, which is meant to design effective strategies at either the government- or the research institutes-level. In this study we have used quantitative methods to examine and establish the trends in stem-cell research. Specifically, we conducted a five-year bibliometric analysis on stem-cell related publications focusing on the United States of America (USA), the United Kingdom (UK) and Japan starting from 2006 to 2010. A universal record for stem-cell related articles published in this period indicates a steady increase of research articles with the USA taking the lead over the UK and Japan. Using co-citation clustering analysis, we further investigated research areas where each of the three countries dominates. We found 155, 24 and 29 stem-cell research competencies for the USA, UK and Japan, respectively. However, the average growth rate of the UK competencies in article number was higher than the rest while, the average number of the citations per publication in competencies from USA was significantly higher than those from the other two countries. We further examined research disciplines of the articles in competencies to understand the major fields of each country in detail, and observed clear differences in research strengths between these countries: stem-cell research in USA was more strong in medical specialties and infectious diseases such as clinical cancer research, immunology and leukemia; in Japan it was more favored by chemistry-related researches such as macromolecules/polymers, pharmaceutical research and food chemistry; UK on the other hand had strengths in engineering and social science field such as data mining, philosophy/psychology, nutrition and human resource management. Conclusively, our findings suggest that these results provide a better understanding of the nation's trends and interdisciplinary strengths

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in the stem-cell research field, which can be used as fundamental information to design national scientific and technological policies and institutional research plans.

Regeneration Mechanisms

Poster Board Number: T-2291

REGENERATION OF AMPUTATED ZEBRAFISH FIN RAYS FROM *DE NOVO* OSTEOBLASTS

Singh, Sumeet Pal, Holdway, Jennifer E., Poss, Kenneth D.
Department of Cell Biology and Howard Hughes Medical Institute, Duke University Medical School, Durham, NC, USA

Determining the cellular source of new skeletal elements is critical for understanding appendage regeneration in amphibians and fish. Recent lineage-tracing studies indicated that zebrafish fin ray bone regenerates through the de-differentiation and proliferation of spared osteoblasts, with limited if any contribution from other cell types. Here, we examined the requirement for this mechanism by using genetic ablation techniques to destroy virtually all skeletal osteoblasts in adult zebrafish fins. Animals survived this injury and restored the osteoblast population within two weeks. Moreover, amputated fins depleted of osteoblasts regenerated new fin ray structures at rates indistinguishable from fins possessing a resident osteoblast population. Inducible genetic fate-mapping confirmed that new bone cells do not arise from de-differentiated osteoblasts under these conditions. Our findings demonstrate diversity in the cellular origins of appendage bone, and reveal that *de novo* osteoblasts can fully support the regeneration of amputated zebrafish fins.

Poster Board Number: T-2292

REGENERATION OF AMPUTATED ZEBRAFISH FIN RAYS FROM *DE NOVO* OSTEOBLASTS

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Poster Board Number: T-2293

GENE-MODIFIED MESENCHYMAL STROMAL CELLS FOR CHRONIC STROKE, MECHANISM OF ACTION AND TRANSLATION TO THE CLINIC

Case, Casey
SanBio, Mountian View, CA, USA

SB623 is an allogeneic adult stem cell product derived from mesenchymal stomal cells (MSCs) by transient transfection with Notch-1. It has proven effective in a variety of preclinical models of neurodegenerative diseases including chronic stroke, traumatic brain injury and Parkinson's disease. SB623 is not cell replacement therapy. It works by mechanisms including trophic support, production of beneficial extracellular matrix and immuno-modulation. A clinical trial has been initiated in patients with stable stroke deficits. This talk will focus on recent findings concerning the mechanism of action and the translation of this product from bench to clinic.

Poster Board Number: T-2294

INNERVATION OF PERIPHERAL AND CENTRAL AUDITORY TISSUES BY HUMAN EMBRYONIC STEM CELL-DERIVED NEURONS *IN VITRO*

Hyakumura, Tomoko[1], Dottori, Mirella[2], Needham, Karina[1], Nayagam, Bryony A.[1]
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The loss of sensory hair cells and auditory neurons that occurs with profound hearing loss is irreversible, and auditory neurons are also observed to progressively degenerate as the period of deafness increases. Stem cell therapy for replacement of lost auditory neurons thereby offers a potential approach to improve the hearing of profoundly deaf individuals. It has been shown that human embryonic stem cell-derived neural progenitors (hESC-NP) can be generated *in vitro* by treatment with Noggin and Y27632, and these neural progenitors can innervate early post-natal cochlear explants. Importantly, for stem cell-derived neurons to improve function in the damaged auditory system, they must be capable of forming functional synapses on target tissue. This project aims to investigate the formation of synaptic connections between hESC-NP and their peripheral and central targets in the auditory system, using *in vitro* models. To examine peripheral innervation, hESC-NP were co-cultured with hair cell explants from post-natal day 2-6 rats (n=18), for 1-2 weeks *in vitro*. Central innervation was examined using co-cultures of hESC-NP with post-natal day 10-12 rat cochlear nucleus slice (n=8), for 2-3 weeks *in vitro*. In both *in vitro* assays, hESC-NP were capable of innervating the target tissue and forming pre-synaptic terminals on target tissue. More specifically, hESC-NP extended processes toward and along the rows of sensory hair cells in all peripheral co-cultures. In addition, extensive numbers of synapsin 1-positive pre-synaptic terminals were detected between hair cells and hESC-NP by 2 weeks *in vitro*. Similarly, we observed extensive innervation of cochlear nucleus slice after 2 weeks co-culture, including many synapsin 1-positive pre-synaptic terminals between hESC-NP and cochlear nucleus tissue. These results illustrate that hESC-NP can innervate peripheral and central target tissues in the developing auditory system and are capable of immature synapse formation by 2 weeks in co-culture. The ability of hESC-NP to synapse on target tissues *in vitro* may depend on both the time in culture and the age of the tissue used. Future investigations using this assay will be directed toward further characterization of the ultrastructure of the new synapses formed, and the

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timing of synapse formation between hESC-NP and their peripheral (hair cell) and central (cochlear nucleus) targets *in vitro*.

Poster Board Number: T-2295

EFFECT OF CTX0E03 HUMAN NEURAL STEM CELLS ON THE TIME COURSE OF SKELETAL MUSCLE REPAIR AND GENE EXPRESSION FOLLOWING HIND LIMB ISCHEMIA IN MICE

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We have previously reported that intramuscular injection of CTX0E03 human neural stem cells (hNSCs) into a mouse model of hind limb ischemia results in a highly significant therapeutic improvement in blood flow. Increased neovascularisation was afforded in the hNSC treated muscle without evidence of long term cell survival. We hypothesized therefore that hNSC treatment may exert an effect on early host muscle responses post-ischemia and upstream of the end point effects reported to date. Skeletal muscle demonstrates rapid degenerative and regenerative phases following injury. In particular, responses of the innate immune system including cellular recruitment and release of pro-inflammatory cytokines have been implicated in the success of skeletal muscle regeneration. Briefly, CD1 mice (8 per time point) underwent unilateral hind limb ischemia followed by immediate injection of either, vehicle or CTX0E03 hNSCs (300K) into the ischemic adductor muscle. Ischemic and contralateral adductor muscles were collected, at 30 minutes, 4, 24 and 72 hr and 7 days post ischemic insult and treatment. To determine the effect of hNSC treatment on these early inflammatory host responses in adductor muscle tissue we investigated cell types, cytokines, chemokines and growth factors associated with the key cellular and molecular events of skeletal muscle repair using histology and an OpenArray™ pathways mouse inflammation panel (Invitrogen). Routine Hematoxylin and Eosin (H&E) staining clearly demonstrated pathologies consistent with muscle degeneration and regeneration including necrosis, recruitment of inflammatory cells and the presence of myocytes with centralised nuclei. Fluorescence immunohistochemistry (IHC) performed using a panel of markers directed against inflammatory cell types, pro-inflammatory cytokines and markers of angiogenesis demonstrated elevation of some of these markers which was attributed to the ischemia. At the earlier time points intramuscular injection of hNSCs significantly increased the expression of the inflammatory cytokine Interleukin-1β (IL1β), CD29, CD44 (mesenchymal cell markers) and Von Willebrand factor. hNSC survival was confirmed up to 72 hr post implantation; hNSCs were lost by apoptosis in a time-dependent manner. OpenArrays were carried out at 0, 4, 24, 72 hr and 7 days post-treatment. In cell injected ischemic muscles, compared with vehicle injected ones, the greatest number of genes showing up-regulation was observed at 4hr, followed by down regulation at 24 hr. Overall, cell treated ischemic muscles showed faster up-regulation of genes involved in angiogenesis, such as CCL11, CCL2, CXCL1, CXCL5, IGF1, IL1β, IL6, HGF, HIF1α, bFGF, VEGFA, and VEGFC, compared to vehicle treated muscles. In summary, we have shown that hNSC treatment transiently elevates host innate immune and angiogenic responses and accelerates regeneration of muscle in a mouse model of limb ischemia.

Poster Board Number: T-2296

SOX2-POSITIVE ADULT MOUSE STEM CELLS GIVE RISE TO ALL EPITHELIAL CELL LINEAGES DURING TOOTH RENEWAL

Juuri, Emma[1], Saito, Kan[1], Ahtiainen, Laura[1], Tummers, Mark[1], Seidel, Kerstin[2], Klein, Ophir[2], Hochedlinger, Konrad[3], Thesleff, Irma[1], Michon, Frederic[1]

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Putative stem cells have been localized in the stellate reticulum of the continuously growing mouse incisor by label-retaining experiments. Specific markers for stem cells that give rise to dental epithelial cell lineages, however, are still unknown. The aim of this study was to find such markers. To achieve this, we defined the genes that are specifically expressed in the incisor cervical loop by microarray and localized their expression by *in situ* hybridization. *Sox2* was specifically expressed in a cell population in the labial cervical loop that is known to give rise to the enamel-producing ameloblasts. By lineage tracing approach, we revealed that in addition to ameloblasts, the Sox2+ epithelial stem cells renew also all other epithelial cell lineages of the incisor. Additionally, we found that the early progeny of Sox2+ stem cells transiently express *Sfrp5*. The discovery of Sox2 as a marker for epithelial stem cell and Sfrp5 for progenitor populations is an important step towards developing a complete bioengineered tooth. Our study provides valuable new insights into the role of stem cells in tooth development and renewal with a potential impact on cell biology, stem cell research, and medicine.

Poster Board Number: T-2297

IDENTIFICATION OF FUNCTIONAL THYMIC EPITHELIAL PROGENITOR CELLS IN THE ADULT MURINE THYMUS

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Thymic epithelial cells play a vital role in the instruction of T cell development, differentiation and tolerance induction. Thymic function and naive T cell output is at optimum levels during the early years of life, however, declines sharply with the onset of puberty (thymic atrophy). This can have a profound impact on immune system function, particular in the aged or following cytoablative regimes such as chemotherapy and irradiation in cancer treatments. Potential methods of thymic regeneration from pre-clinical studies include sex steroid ablation and administering growth factors such as keratinocyte growth factor, growth hormone or interleukin-7. Whilst some of these approaches are in early clinical trials, ideal T cell recovery levels have not yet been met and patient responsiveness, particularly in those with severe thymic atrophy, cannot be guaranteed. Therefore, an alternative approach would be to activate resident thymic epithelial progenitor cells to aid in the regeneration of the thymus in states of dysfunction. Using flow cytometric analysis, a relatively quiescent thymic epithelial progenitor was deduced based on long-term BrdU label-retention over a 6 month chase. Functional studies using reaggregate thymic organ cultures found that these putative progenitors were the only population capable of forming a thymic graft when placed under the kidney capsule of recipient mice, contributing to both medullary and corti-

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cal thymic lineages. Furthermore, 3-dimensional epithelial cultures showed that this putative progenitor had increased colony-forming capacity over other thymic epithelial populations. In summary, we have established that a thymic epithelial population of cortical lineage shows enriched progenitor/stem cell potential capable of differentiation and self-renewal.

Poster Board Number: T-2298

QUIESCENT PRIMITIVE NEURAL STEM CELLS IN THE ADULT MOUSE BRAIN REPOPULATE ABLATED DEFINITIVE NEURAL STEM CELLS

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We recently identified a novel population of LIF-dependant neural stem cells (NSCs) in the adult mouse brain, termed adult-derived primitive NSCs (AdpNSCs). AdpNSCs comprise a rare population of GFAP-negative cells that are similar to embryonic primitive NSCs, as they give rise to LIF-dependent neurospheres in culture. AdpNSCs can be passaged *in vitro* to self-renew or give rise to definitive (d) NSCs, which are GFAP-positive and EGF- and FGF-dependent. AdpNSCs were shown to express low levels of Oct4 and contribute to chimeric blastocysts, both markers of pluripotency not exhibited by adult dNSCs. Unlike dNSCs, AdpNSCs depend on Oct4 expression in the adult brain. Transgenic mice with a floxed Oct4 gene that is excised when exposed to Cre recombinase expressed under the Sox1 promoter were used to assess the requirement for Oct4 in AdpNSCs. Most interesting, the forebrain of adult mice homozygous for the deleted floxed Oct4 allele no longer gave rise to any LIF-dependent neurospheres, while EGF- and FGF-dependent neurospheres were not affected by loss of Oct4. This indicates a requirement for Oct4 expression in AdpNSCs, and provides a key model for an AdpNSC-null mouse. Next, we sought to determine whether AdpNSCs are required for repopulation of dNSCs following ablation of dNSCs with AraC infusion. We ablated dNSCs and downstream neural progenitors in the excised Oct4 transgenic and control mice using a 14-day AraC infusion. After the 14-day AraC infusion and a 14-day recovery after AraC, we observed that the EGF- and FGF-dependent neurospheres did not return in mice lacking Oct4 expression whereas control mice had repopulated dNSCs to 40% baseline conditions at this time point. This suggests that AdpNSCs are essential for repopulation of dNSCs. We are continuing this experiment with longer survival times to further confirm this observation. Furthermore, we took advantage of the Histone2B-GFP transgenic line to perform label retention studies in AdpNSCs. In this model, all cells are labeled when exposed to doxycycline and then dilute their label as they divide, thereby leaving quiescent cells labeled over long chase periods. We observed that after 1-month chase, AdpNSCs retained the same degree of labeling, whereas over half the labeled dNSCs had diluted out their label. This indicates that AdpNSCs are a quiescent cell population in the adult mouse brain. Together, these experiments indicate that AdpNSCs are a quiescent population upstream of dNSCs in the adult mouse brain, capable of proliferation after removal of the dNSC population to reconstitute the neural lineage.

Poster Board Number: T-2299

ENDOVENOUS ADMINISTRATION OF MESENCHYMAL STROMAL CELLS IMPROVES KIDNEY FUNCTION IN MICE WITH TYPE 2 DIABETES MELLITUS INDUCED BY HIGH FAT DIET FEEDING.

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Diabetes mellitus (DM) is one of the current main threats to public health. According to its etiology, DM is classified as: i) Type 1 diabetes mellitus (T1DM), due to the autoimmune destruction of pancreatic β-cells, which leads to an insulin deficiency and ii) Type 2 diabetes mellitus (T2DM), a metabolic disorder whose principal characteristic is the presence of insulin resistance, together with an impaired insulin production. The most detrimental complication of DM is diabetic nephropathy (DN), a clinical syndrome comprised of kidney damage and increased risk for cardiovascular disease. DN associates with functional and histological changes that include: albuminuria, mesangial expansion, glomerulosclerosis and tubulointerstitial fibrosis. To date there is no cure to DN; patient`s management comprises the use of drugs to control hyperglycemia and blood pressure. If required, hemodialysis is prescribed; unfortunately, those treatments only help to slow DN progression. Multipotent mesenchymal stromal cells also referred as mesenchymal stem cells (MSCs) are one of the most promising tool to manage DN progression, not only because they can be safely transplanted in human patients but also due to their proved renoprotective potential. The later, has been attributed to the capacity of MSCs to: i) reduce oxidative stress, ii) modulate chronic inflammation and macrophage infiltration, iii) avoid/revert fibrosis, iv) secrete trophic factors, and v) differentiate into pericytes, mesangial and tubular cells. Recently, we have shown that the intravenous administration of MSCs into mice with DN derived from T1DM, prevents kidney damage. At present, our research aim was to evaluate whether the MSCs renoprotective effect depends on DM etiology (type 1 vs type 2). The complex etiology of T2DM makes it difficult to perfectly mimic this disorder in experimental models; however C57BL/6 mice fed with high-fat diet containing 60% kcal. satured fat (HFD) develop renal functional and histopathological abnormalities similar to those observed in patients with obesity, metabolic syndrome or T2DM onset. Here, C57BL6 mice were exclusively fed with HFD and 33 weeks later, separated in two groups. One received two times 0.5x106 MSCGFP that have been *ex vivo* expanded from transgenic mice that constitutively express GFP (MSC-treated). The other group received vehicle (untreated). Both groups continued to eat HFD all along the study period (50 weeks). Renal failure did not progress in MSC-treated T2DM mice, while in untreated T2DM mice albuminuria gradually increased. These changes were correlated with morphological alterations and glomerulosclerosis index. Two and 17 weeks post-MSC administration, biodistribution was evaluated by flow cytometry. We found donor cells in the kidney of mice with T2DM but not in normal mice. The observed renoprotective effect of MSCs was not related to a reversion in the metabolic syndrome since MSC-treated mice kept hyperglycemic, hyperinsulinemic, insulin resistant and hypercholesterolemic along the study period. Although donor cells were found in the kidney of treated mice, they scarcity suggest that MSCs systemic administration improve kidney function not by functional complementation from direct differentiation parenchyma. Probably is the consequence of microenvironment changes. Additional experiments are in course

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to clarify the mechanisms associated to the observed therapeutic effect. Supported by FONDECYT 1120133 to ME

Poster Board Number: T-2300

OLFACTORY DERIVED NEURAL PROGENITOR CELLS TRANSPLANTATION IN A RAT INTRACEREBRAL HEMORRHAGE MODEL

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Background: Olfactory epithelium is easily accessible neural tissue and harbors abundant neural progenitor cells which produce new neurons every day in response to various environmental stimuli. We tried to investigate neuronal regeneration effect of olfactory derived neural progenitor cells (ONPC) transplantation in intracerebral hemorrhage model. Methods: We harvested ONPCs from human nasal olfactory mucosa culture from DMEM/HAM F12 media, followed by DMEM/HAM F12 supplemented with insulin, transferrin, selenium (ITS-X 1%), EGF, and FGF2 for four days. We transplanted 0.3 million ONPCs in a collagenase induced rat intracerebral hemorrhage model, and functional outcome were measured every week until fifth week. In vitro and *in vivo* cell marker studies were performed to evaluate cellular phenotype of ONPCs. Results: In vitro cell marker studies before transplantation showed that ONPC are combined with doublecortin, microtubule associated protein (MAP2), and GABA, but not with GFAP or NeuN, suggesting immature neuronal progenitor cell morphology. Transplantation of ONPCs attenuated functional deficit after intracerebral hemorrhage model after five weeks, and *in vivo* cell marker study five weeks after transplantation showed decrement of immature neuronal markers. Discussion: Olfactory epithelium derived ONPC attenuated neurological deficit after intracerebral hemorrhage presumably by differentiation of neuronal lineage.

Poster Board Number: T-2301

A TWO-STEP DIRECTED DIFFERENTIATION OF RAT EMBRYONIC STEM CELLS USING FGF2 AND LAMININ AND SONIC HEDGEHOG RESULTS IN MOTOR NEURONS WITH AN EXPRESSION PROFILE SIMILAR TO MOTOR NEURONS DERIVED FROM PRIMARY NPCS DERIVED FROM THE ADULT CERVICAL RAT SPINAL CORD

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New to biomedical research, rat embryonic stem cells (ESCs) differentiate to every adult cell type offering the potential as a component of a multi-rodent model to study differences amongst tissues. We have previously derived rat ESCs from various strains. To create an *in vitro* model of stimulating central nervous system motor neuron neogenesis, we first differentiated rat ESCs to neural progenitor (NPC)-like cells. Neural progenitors have the capacity to differentiate into neurons and nonneural support cells. NPC-like cells were produced from rat ESCs through administration of basic fibroblast growth factor (FGF2) and laminin on poly-L-ornithine and laminin coated or matrigel coated cell culture plates. Subsequently, we differentiated the NPC-like cells and adult rat cervical spinal cord derived primary NPCs to motor neurons with sonic hedgehog (Shh). Differentiation was accomplished through two ways. One involved expanding NPC-like cells in monolayer culture. The other progressed through neurospheres. Motor neuron cultures were analyzed for expression of Nestin, Pax6, Tubb3, Irx3, Nkx2.2, HB9, ISL1 and SMI-32. This protocol was also applied to primary NPCs isolated from the lumbar region of the adult rat spinal cord. Stimulating *in vivo* motor neuron regeneration after spinal cord injury remains elusive, and, therefore, the development of an *in vitro* model to test compounds that promote motor neuron regeneration in the presence of oligodendrocytes, Schwann or Olfactory bulb ensheathing cells may be informative.

Poster Board Number: T-2302

SONIC HEDGEHOG MEDIATES ENDOGENOUS-EXOGENOUS NEURAL STEM CELL SYNERGY AND NEUROPROTECTION IN THE PARKINSONIAN RAT

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Neuroprotective and neurorescue effects after neural stem/precursor cell (NPC) transplantation have been reported, but the mechanisms underlying such phenomena are not well understood. Our recent findings in a rat Parkinson's disease (PD) model indicate that transplantation of NPCs before a 6-hydroxydopamine (6-OHDA) insult can result in nigrostriatal protection which is associated with endogenous NPC proliferation, migration and neurogenesis suggesting a 'synergy' between transplanted (exogenous) and endogenous NPC actions *(Madhavan et al, 2009; J. Comp. Neurol; Madhavan et al, Neuropharmacology, 2010)*. In addition, the transplanted NPCs expressed certain growth factors [including glial derived neurotrophic factor (GDNF), and sonic hedgehog (SHH)] providing a potential molecular basis for the observed phenomenon. We have investigated mechanisms underlying the observed NPC-mediated neuroprotection and neurogenesis by examining roles of (a) host endogenous NPCs and (b) abovementioned graft-expressed factors. With respect to the endogenous NPCs, we have established that the host neural precursors not only respond to the transplanted NPCs but in fact contribute to dopamine system neuroprotection (*Madhavan et al, European Journal of Neuroscience, 2012)*. The current study focuses on the grafted NPCs and their 'chaperone effects', and in particular examines the role of GDNF and SHH using RNA interference. Specifically, NPCs in which either GDNF, SHH, or both had been silenced were transplanted into host rats to determine whether or not they contribute to the observed NPC-mediated neuroprotection and endogenous response to transplantation. Histological and behavioral analyses indicate that GDNF silencing significantly reduced NPC-mediated neuroprotection but not neurogenesis. On the other hand, knock-down of SHH, or a combination of GDNF and SHH caused a profound decrease in both graft-mediated neuroprotection and endogenous NPC activation (proliferation, migration and neurogenesis), suggesting that SHH was a key molecule contributing to NPC mediated therapeutic effects. Overall, the studies help determine some of the micro-environmental signals fundamental to neural precursor based neuroprotection, and contribute towards the development of novel stem cell based therapies for PD.

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Poster Board Number: T-2303

THREE CHEMOKINES, SDF1A, MCP-1 AND HEPATOCYTE GROWTH FACTOR ATTRACT TRANSPLANTED NEURAL STEM CELLS DURING NEUROINFLAMMATION

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Neural stem/precursor cells (NSCs) possess powerful immune-regulatory and neurotrophic properties that underlie their therapeutic effects after transplantation in animal models of multiple sclerosis (MS). Since MS is a multifocal disease, NSCs need to migrate into the multiple lesions in order to exert their therapeutic effects. Therefore, cell migration is a crucial element in regenerative processes in MS, dictating the route of delivery, when cell transplantation is employed. We have previously shown that inflammation triggers migration of multipotential NSCs into white matter tracts of experimental autoimmune encephalomyelitis (EAE) rodents, a widely used model of MS. Here we investigated the molecular basis of this attraction. First, inflammation-triggered NSC migration into white matter tracts was dependent on a motile NSC phenotype, induced by epidermal growth factor (EGF). Specifically, migration of FGF2-expanded NSCs was observed in only 10% of EAE mice, as compared to 47.5% of EAE mice transplanted with FGF2+EGF -expanded NSCs. A similar effect was found in an *in vitro* migration assay. Then, we examined tissue factors that attract transplanted NSC migration during EAE. Three chemokines, Stromal Derived Factor-1α (CXCL12), Monocyte Chemoattractant Protein-1 (CCL2) and Hepatocyte Growth Factor, were induced in tissue microglia and astrocytes during EAE. Blocking of each of their specific receptors (CXCR4, CCR2 and c-Met, respectively) on NSCs prior to transplantation, caused partial inhibition of cell migration in the corpus callosum of EAE mice. Blocking all three receptors caused a profound 80% inhibition of transplanted NSC migration. These findings were replicated in two different EAE models. Thus, three glial derived chemokines and their receptors account for most of the tissue-derived attraction of transplanted NPCs into inflamed white matter tracts during EAE. These findings identify inflammatory signals that are involved in putting regenerative processes in motion, and suggest potential therapeutic targets for regenerative medicine in multiple sclerosis.

Poster Board Number: T-2304

REGENERATION OF THE DENTAL PULP VIA AMPLIFICATION OF AN ENDOGENOUS WNT SIGNAL

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Adult tissues contain stem cells, which are activated by injury and contribute to the repair of damaged tissues. Dental pulp is one such tissue, but the extent of stem cell activation occurs following injury is unknown, and whether a strategy could be devised that induces dentin regeneration remains untested. Using *Axin2*$^{LacZ/+}$ reporter mice, we identified odontoblasts as being responsive to an endogenous Wnt signal that is produced in the dental pulp itself. When the tooth is injured, the dental pulp responds by up regulating Wnt target genes. We tested the consequences of amplifying the endogenous Wnt signal. To do this we employed *Axin2*$^{LacZ/LacZ}$ mice, where loss of both alleles of the negative Wnt regulator Axin2 leads to an enhanced Wnt responsiveness that nonetheless retains its ligand-dependency. In control *Axin2*$^{LacZ/+}$ mice the injury site was filled with an inflammatory infiltrate that gradually resolved. At no time during the post-surgical period did we detect evidence of a repair/regenerative response. On post-surgery day 7 the injury site in *Axin2*$^{LacZ/+}$ mice was filled with undifferentiated fibroblastic cells and some blood vessels. On post-surgery day 10 the appearance of the injury site had not changed appreciably. In *Axin2*$^{LacZ/LacZ}$ mice, however, exhibited a dramatically different response: on post-surgery day 7 the injury site was filled with fibroblastic cells and blood vessels and cells lining the injury site had achieved an osteoblast-like morphology. On post-surgery day 10 the injury site in *Axin2*$^{LacZ/LacZ}$ mice was filled with a newly formed mineralized tissue. This tissue was localized exclusively at the area of irritation, and had irregular, reduced dentinal tubule architecture. The mineralized tissue stained positive to collagen type I, and thus constituted tertiary or reparative dentin. These data demonstrate that enhancing the endogenous Wnt signal results in a superior regenerative response from dental pulp stem cells. We then employed a biochemical strategy that mimicked the transient elevation in endogenous Wnt signaling observed in *Axin2*$^{LacZ/LacZ}$ mice, by delivering to the injured pulp a liposomal formulation of Wnt3a protein that maintains the biological activity of the hydrophobic protein. The regenerative response elicited by this treatment strongly suggests that by amplifying the body's natural response to injury we can induce a regenerative response in the dental pulp.

Poster Board Number: T-2305

TWO-PHOTON MICROSCOPY TO CAPTURE LIVE CELL BEHAVIOR IN THE HAIR FOLLICLE STEM CELL NICHE

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Stem cells and niche components are responsible for the timely orchestration of the regeneration process that leads to highly organized tissues. Despite recent progress in our understanding of stem cell biology, the dynamic interaction between stem cells and the niche is not well understood. A current challenge in the field is having access to a well-defined stem cell niche in which the orderly development of stem cells can be observed, characterized and manipulated *in vivo*. To learn which dynamic behaviors occur during mammalian regeneration, we took advantage of the unique accessibility of the skin hair follicle and set up a novel imaging approach to study a physiological regeneration process non-invasively by two-photon microscopy. By these means, we have studied the behavior of the epithelial stem cells and their progeny during physiological hair regeneration and how the mesenchymal niche influences their behavior. Consistent with earlier studies, stem cells are quiescent during initial stages of hair regeneration, whereas the progeny are more prone to divide. Moreover, stem cell progeny cell divisions are spatially and temporally coordinated within follicles. In addition to cell divisions, coordinated cell movements within the progeny allow hair follicle rapid expansion. Finally, we demonstrate the requirement for the mesenchymal niche for hair regeneration through targeted cell ablation and long-term tracking of live hair follicles. Thus, we have established an *in vivo* approach that has led to the discovery of unpredicted mechanisms of growth regulation, and enabled us to precisely investigate functional requirements of stem cell niche components during the process of physiological regeneration.

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Poster Board Number: T-2306

SOX2 MARKS EPITHELIAL COMPETENCE TO GENERATE TEETH

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Tooth renewal takes place by budding from the epithelium of the predecessor tooth. Mammalian molars develop in succession from a posterior extension of the epithelial dental lamina associated with the previously formed molar. In tooth replacement each individual replacement tooth is initiated as an extension of the dental lamina from the lingual side of the predecessor. Tooth replacement capacity varies between different species from one round in mammals to lifelong replacement in reptiles and fish. For the successional generation of new teeth to be possible there has to be stem cells in the epithelium that retain the competence for renewal. So far the localization and the molecular identity of these cells in mammals have been uncharacterized. Understanding the mechanisms of tooth renewal requires identification of the competent stem cells. Transcription factor Sox2 has been shown to regulate stem cell maintenance as well as iPS cell induction. We have identified Sox2 as a marker to identify the competent cells in the dental epithelium. We have localized *Sox2* expression in tooth replacement of different species such as the ferret (*Mustela putorius furo*), where Sox2 positive cells are seen in the lingual epithelium giving rise to the permanent teeth. In addition we have localized Sox2 in the dental epithelium of mutant mice where overactivation of Wnt signaling leads to continuous tooth renewal. In the successional development of molars *Sox2* expression marks the budding epithelium. Our data suggest that the Sox2 positive dental epithelial cells associated with tooth replacement and posterior molar addition may represent stem cells for successional tooth renewal.

Poster Board Number: T-2307

PROSTAGLANDIN E2 PROMOTES POST-INFARCTION CARDIOMYOCYTE REPLENISHMENT BY ENDOGENOUS SCA-1 STEM CELLS

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In mammals, adult cardiomyocytes are capable of regenerating themselves following injury or during normal lifespan. Furthermore, the adult stem cells possessing cardiomyocyte differentiation ability have been identified. Nevertheless, it remains unknown how these stem cells are stimulated to repair the heart after injury. In our study, we used adult cardiomyocyte-specific fate-mapping system (a-MHC:MerCreMer crossbreeding Z/EG) to examine the time course of stem cell-driven cardiomyocyte replenishment after myocardial infarction (MI) in mice. We found that formation of new cardiomyocytes took place at the border zone within 7 days and saturated on day 10 post-MI. Further analyses indicated that administration of anti-inflammatory drugs, indomethacin and celecoxib, inhibiting cyclooxygenase (COX)-2 diminished cardiomyocyte regeneration after infarction. Moreover, such inhibition was only effective when the drug was administrated within 5 days post-MI, suggesting an early inflammatory signal is required to activate endogenous stem cell-dependent cardiomyocyte replenishment. In addition, treating the mice with the COX-2 downstream product prostaglandin E2 (PGE2) not only improved efficiency of stem cell-driven cardiomyocyte replenishment but also rescued indomethacin-mediated inhibition of cell regeneration. Administration of PGI2, another COX-2 product, had no such effects. Quantitative PCR analysis of stem cell marker expression revealed that Sca-1 level changed in response to PGE2, in which its level elevated after treatment on day 3 post-MI. Immunostaining showed presence of the Sca-1+ cells co-expressing mature cardiomyocyte maker cardiac troponin T (cTnT) at the injured site or border zone of the MI heart. Flow cytomeric analysis indicated that the Sca-1+ cells expressed Cre and became GFP+ on day 3 post-MI. The number of Sca-1+/cTnT+ cells and the percentage of Sca-1+/GFP+ cells were found to increase upon PGE2 treatment on day 3 after MI. In addition, these cells were not generated as a consequence of cell fusion. To examine if PGE2 directly acted on the Sca-1+ cells, the cells were sorted and quantitative PCR was performed to analyze expression of the PGE2 receptors EP1-EP4 on day 3 after MI. We observed that PGE2 not only induced expression of the EP2 and EP4 receptors but also the level of cardiac progenitor cell marker Nkx-2.5 in the Sca-1+ cells. Moreover, PGE2 treatment stabilized β-catenine protein level in these cells. In *in vitro* culturing, PGE2 treatment promoted cTnT expression of the Sca-1+ cells. These findings suggest that PGE2 may directly act on the Sca-1+ cells via EP2/4 receptors to modulate β-catenine pathway for cardiomyocyte differentiation. Taken together, we demonstrate that the early inflammatory modulator PGE2 promotes cardiomyocyte differentiation from cardiac Sca-1+ cells, and this may be translated for clinical application.

Poster Board Number: T-2308

LEUKEMIA INHIBITORY FACTOR ADMINISTRATION ENHANCES ENDOGENOUS CARDIOMYOCYTE REGENERATION AFTER MYOCARDIAL INFARCTION BY ACTIVATING CARDIAC SIDE POPULATION CELLS: A GENETIC FATE-MAPPING STUDY

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Cardiac stem or precursor cells have the potential to regenerate cardiomyocytes, but their contribution to regeneration and their role in the efficacy of cardioprotective drugs remain controversial. We have previously reported the ability of cardiac side population (SP) cells to differentiate into cardiomyocytes after being transplanted. In this study, using a genetic fate-mapping model, we show that leukemia inhibitory factor (LIF) influences SP cell proliferation and stimulates endogenous cardiomyocyte renewal after myocardial infarction (MI). We generated MerCreMer-LacZ mice, in which more than 99.9% of the cardiomyocytes in the left ventricular field were positive for 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal) immediately after tamoxifen injection. Thus, every Xgal-negative cardiomyocyte was derived from a stem or precursor cell after tamoxifen administration. During normal aging spanning 1 year, the number of Xgal-negative cardiomyocytes in all the mice did not change significantly: 9.8 ± 3.8 and 9.8 ± 5.2 cells per section at 2 weeks and 1 year respectively. However, at 3 months after MI, the MI mice had more Xgal-negative cells than the control mice (57.0 ± 12.0 and 3.0 ± 2.6 cells per section respectively; $P < 0.01$). Xgal-negative cardiomyocytes significantly co-localized with vessels within the MI area and were smaller than Xgal-positive cells (median: 51 vs. 151 μm2). The part of SP cells were shown to be label-retaining cells (LRCs), that differentiated into Xgal-negative cardiomyocytes after MI. To determine which factors induced stem-cell derived

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regeneration, we examined cytokines secreted after MI. LIF expression was 139 times higher in the MI mice than in the control mice, but its expression rapidly decreased within 1 week. We injected the LIF plasmid or phosphate-buffered saline (PBS) at the time of MI to determine the influence of LIF. In the LIF plasmid-injected MI mice, the LIF serum concentration peaked at 1 week and could be detected until 1 month. At 1 month after MI, the MI+LIF group had a greater number of Xgal-negative cells than the MI+PBS group (118.6 ± 51.5 and 37.0 ± 5.5 cells per section; $P < 0.05$). Echocardiography showed significant recovery of functional shortening in the LIF-treated MI group only. Finally, we examined the effect of LIF on SP cells. Quantitative RT-PCR confirmed that SP cells expressed a considerable number of LIF receptors. Immunofluorescence analysis demonstrated that the percentage of phosphorylated signal transducers and activators of transcription 3-positive and cyclin D1-positive SP cells increased after LIF stimulation *in vitro*. On BrdU administration at 1 week after MI, the percentage of BrdU-positive SP cells in LIF-treated mice was 59%, while it was 35% in PBS-treated mice, suggesting that LIF influenced SP cell proliferation. The percentage of Ki67 and phosphorylated histon 3 positive SP cells were also higher in LIF-treated mice. These results suggest that LIF stimulates stem cell-derived cardiomyocyte regeneration in part by activating SP cells after MI. We believe that our findings form the basis for future therapeutic cardiogenesis strategies.

Poster Board Number: T-2309

THE ORIGIN OF DIVIDING CELLS FOR HEAD REGENERATION IN THE HEMICHORDATE, PTYCHODERA FLAVA

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When the body of the marine acorn worm, P. flava, is transected, both pieces regrow the lost parts. The posterior segment grows a new head at it anterior wound. The wound heals in about 2 days and a blastema is evident at the anterior site by 3 days. By 5 or 6 days the blastema takes the shape of a nascent head and in about two weeks grows to full size. The source of the cells and the nature of the biological processes that form the new head in P. flava have not been studied extensively. In hydra and planaria, animals know for extensive regenerative capacity, there is a population of stem cells which are activated and mobilized to form the regenerate. In another classical model of regeneration, limb regrowth in vertebrate amphibians, the blastema cells seem to be recruited from the differentiated tissue, possibly from adult tissue stem cells or from dedifferentiation of tissue cells in the various tissues of the transected limb stub. Using BrdU immunofluorescence cytochemistry to mark dividing cells, we examined the source of cells that produce the regenerate in P. flava. We transected the worms just posterior to the gill basket, let regeneration proceed for various times and then labeled with BrdU for 6 hours. Before cutting the animal, there is a considerable population of cells along the gut epithelium, especially in the liver region of the animal, that incorporate BrdU. This population of dividing gut cells does not seem to change in number or character during regeneration. There are some BrdU labeled cells scattered in other regions of the animal's body. Some of these occur in the epidermal epithelium and along the basement membrane under this epithelium. It is this population of cells associated with the epidermal epithelium that increases with regeneration. By day 3 after the cut, there are abundant BrdU labeled cells associated with the epidermis within a few millimeters of the cut, both in the epithelium itself and under the basement membrane of the epithelium. This population of cells is more abundant nearer the site of the cut and in the dorsal epithelium compared to the ventral epithelium. By day 3 there is a clear blastema and a large proportion of the cells in the blastema incorporate BrdU during the labeling period. Labeling of cells under the basement membrane, in the epithelium near the regenerating blastema and in the regenerate itself continues for about two weeks as the new head is differentiated. During all there processes, there is no indication of remodeling of the original tissue behind the regenerating blastema. These data suggest that there is a population of cells associated with the worm epidermis that is activated to divide and mobilized to form the blastema and the regenerate.Supported by the Pacific Biosciences Research Center Biomedical Fund (UHF#12-209-04)

Poster Board Number: T-2310

HEDGEHOG SIGNALING PATHWAY ACTIVATION IN THE HEMATOPOIETIC NICHE STROMAL CELLS

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The Hedgehog (Hh) signaling pathway plays key regulatory roles during development, adult tissue homeostasis and cancer. Recent studies further identified the importance of Hh signaling during regenerative proliferation of stem cells in response to tissue injury. However, the mechanistic basis of pathway activities within certain specific cell types in their native microenvironment remains largely unknown. Hematopoietic stem cells (HSCs) require stromal support for their maintenance at steady state and during injury-induced regeneration. Several developmental conserved pathways are implicated in the maintenance of hematopoietic cells and their cell fate decisions. Importantly, interactions between the hematopoietic cells and their microenvironment in terms of specific regulatory pathways are still poorly characterized. Here we investigate the role of Hh signaling pathway in hematopoietic stem/progenitor cell niches. Current evidence suggests a model where multiple types of stromal cells are responsible for the maintenance of hematopoietic cells. Using genetic reporters for Hh pathway activation in conjunction with surface markers that can distinguish different subsets of skeletal progenitor cells, we found activation of Hh pathway in specific lineages of cells isolated from postnatal bone tissue. In addition, activation of the Hh pathway diverted cell fate decisions in the stem/progenitor cells during their differentiation process. Since these subsets of stromal cells exhibit differential ability to support hematopoietic stem and progenitor cells, our results implicated important roles of Hh pathway in regulating hematopoietic niches. We will also present our investigations on Hh pathway activities during normal tissue maintenance and injury induced hematopoietic regeneration.

Other

Poster Board Number: T-2311

EMBRYONIC TRANSCRIPTION FACTOR UPREGULATION DURING NORMAL LACTATION AND BREAST ONCOGENESIS

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Mammary stem cells (MaSCs) play important physiological roles during pregnancy and lactation in the female mammary gland. In the resting breast, MaSCs are believed to be in a quiescent state and are represented in scarce numbers. However, during

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pregnancy and lactation these cells become activated in response to hormonally-driven cues, and differentiate into two distinct populations, the basal myoepithelial and the luminal cells. Recent evidence suggests that MaSCs are primary targets of malignant transformation giving rise to aggressive triple negative breast carcinomas. The molecular determinants that drive aberrant self-renewal in these cells are largely unknown. Progress has been limited because of our lack of understanding of normal MaSC biology and the absence of cell culture model systems to propagate and characterize these cells. To discover early determinants of transformation in MaSCs, we used human breastmilk as a novel, non-invasive, and patient-specific source of MaSCs in the activated mammary gland. A multipotent MaSC population was identified in breastmilk, expressing the pluripotency transcription factors (TFs) Oct4, Sox2 and Nanog, known to maintain self-renewal in human embryonic stem cells (hESCs). Upon expansion in feeder cultures, milk-derived MaSCs exhibited the encapsulated ES-like colony morphology. These colonies could be passaged in secondary feeder cultures suggesting clonogeneicity and self-renewal. *In vitro* differentiation assays demonstrated the potential of these cells to give rise to cell lineages from all three germ layers. Propagation of milk-derived MaSCs in spheroid assays resulted in a dramatic up-regulation of TFs known to form an integral part of the complex self-renewal circuitry of hESCs. In contrast, these TFs were not expressed in the resting epithelium and in cultures derived from resting mammoplastic reductions. In accordance with this model, forced ectopic expression of Oct4 in cells from the resting epithelium resulted in aberrant expansion of MaSCs possessing multi-lineage potential and displaying tumor initiating features. Oct4 and targets of Oct4 were examined by IHC and IF across breast cancers and were found enriched in breast cancers associated with pregnancy and lactation. These findings suggest that disruption of controlled expression of these TFs during pregnancy and lactation, failure to silence them during involution, and/or their aberrant upregulation in the resting breast can be at the origin and progression of aggressive breast carcinomas. Our data suggest that milk-derived MaSCs can be used as a non-invasive, personalized source of MaSCs to study molecular determinants of breast cancer.

Poster Board Number: T-2312

BREASTMILK IS A NOVEL SOURCE OF STEM CELLS FROM THE LACTATING BREAST WITH MULTI-LINEAGE DIFFERENTIATION POTENTIAL

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The mammary gland is a dynamic organ that undergoes repeated cycles of proliferation, differentiation and apoptosis in response to hormonal signals initiated during pregnancy and lactation. This unique ability relates to the presence of stem cells in a specialised niche of the mammary gland. Whilst these cells have mostly been studied in mice and in the context of the resting gland, unique insights may be gained by the study of the lactating gland in which these cells are activated and exist in larger numbers, with emphasis on the human gland, for which our knowledge is very limited. We non-invasively accessed stem cells from the human lactating mammary gland via freshly expressed breastmilk. We performed an *ex vivo* analysis of the cellular populations present in breastmilk using phenotypic and functional stem cell and differentiation markers for various lineages. This analysis resulted in the identification of candidate stem cell and progenitor populations in breastmilk. Importantly, a cell population was identified expressing the pluripotency-associated embryonic stem cell markers Oct-4, Sox-2, Nanog, SSEA-4, Tra-1-60 and Tra-1-81. We then localised the identified cell populations in the human lactating mammary gland, using paraffin-embedded sections of normal human lactating tissues. Culturing breastmilk cells in embryonic stem cell and differentiation media, in feeder-free 2D conditions, using feeders, and in 3D conditions, revealed the potential for both self-renewal/clonogeneicity and differentiation into cell types originating from all three germ layers. The data obtained from our *ex vivo*, in situ and in vitro analyses were synthesised into a new cellular hierarchy proposed for the human mammary gland. Moreover, for the first time it is shown that the human lactating gland harbours a stem cell population with embryonic-like stem cell features, which can be easily accessed via breastmilk. Future work is focusing on further characterisation of these cells for applications in regenerative medicine and breast cancer research, as well as for elucidating their role(s) for the breastfed infant.

Poster Board Number: T-2313

HUMAN BONE MARROW STROMAL CELLS SHOW RESISTANCE TO HIGH CALCIUM ENVIRONMENT *IN VITRO*

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Endosteal niche micro-environment within bone marrow is known to have extremely high concentration of calcium, which reaches as high as 40mM locally near resorbing osteoclasts. Because human bone marrow stromal cells (hBMSC) reside in the environment of high calcium concentration, we expected that hBMSCs might have more resistance to high calcium concentration. We examined the effect of various concentrations of calcium on the survival of hBMSCs compared to human dermal fibroblasts (HDF) as a control. Although both of the cells showed decline in the cell number in high calcium media, we found the viability of hBMSCs is significantly higher than that of HDFs cultured in the media containing from 3 to 30mM concentration of calcium by MTT assay. When proportion of apoptotic cells was measured by FACS analysis of propidium iodide-incorporated fraction of cells, proportion of the apoptotic cells increased as the calcium concentration elevated which indicated that the declined cell number in the high calcium media was due to cell apoptosis. While high calcium media severely induced growth arrest of HDFs, this tendency was weakly shown in BMSCs and rather, growth was accelerated by 3~5 mM calcium concentration in BMSCs. These results indicate that hBMSCs are resistant to high calcium concentration and may explain their survival mechanism in endosteal niche environment.

Poster Board Number: T-2314

BANK OF NORMAL AND SPECIFIC MUTATION-CARRYING HUMAN EMBRYONIC STEM CELL LINES

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The Assisted Conception Unit and Centre for Preimplantation Genetic Diagnosis (PGD) at Guy's Hospital in London has the largest and most successful PGD program in the UK, performing around 200 cycles each year. We are currently performing PGD for more than 60 conditions in our unit. Clinically unsuitable embryos, if donated for research by consented couples, were used to derive specific mutation-carrying human embryonic stem (hES) cell lines. The success rate for derivation using fresh post-PGD embryos now

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sits at between 40-50%, which is a significant improvement on our original 20%, and higher than most quoted efficiencies in the literature. We now have derived multiple hES cell lines including these with mutations linked to cystic fibrosis, Huntington's disease, Von Hippel-Lindau syndrome, Wiskott-Aldrich syndrome, myotonic dystrophy type 1, neurofibromatosis type 1, spinal muscular dystrophy and beta thalassemia (www.kcl.ac.uk/medicine/research/divisions/wh/groups/medicine/hescell.aspx). These specific mutation-carrying hES cells can be differentiated into any cell type that displays pathology of the disease and used in drug discovery for screening of new compounds and testing of unacceptable side effects. We also have a number of research and clinical grade normal hES cell lines and our goal is to make these biological tools accessible worldwide to everyone, from researchers to commercial end users.

Poster Board Number: T-2315

RECOVERY INCREASED BY SIMPLE IMPROVEMENT OF THE CONVENTIONAL CRYOPRESERVATION METHOD FOR THE HUMAN ES AND IPS CELLS

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Human embryonic stem cells (hESCs) and human induced pluripotent cells (hiPSCs) have great potential as a source of cells for regenerative medicine and also for other research applications, such as developmental biology, toxicology and drug discovery. Recently, a number of hESCs and hiPSCs have been established in the world. Although most of the procedures share many of the principles of routine cell culture, a number of significant steps exist in the case of hESCs and hiPSCs, such as the cryopreservation.
Both hESCs and hiPSCs have been well-known to be sensitive to the cryopreservation. At this moment, vitrification method has been considered to be preferable for the local storage of hESCs and hiPSCs. However, vitrification method requires practiced hand because cells can be terribly damaged by failure of rapid-warming. Further, if the low temperature is not strictly maintained, cell viability is rapidly decreased. Therefore, when vitrified cell transported, it needs LN2 dry-shippers with careful documentation for the regulatory requirements, resulting in the high costs involved. On the other hand, the conventional slow-freezing method using dimethylsulfoxide (DMSO) yield poor results for hESCs and hiPSCs, such as low levels of recovery or spontaneously differentiation, although it has been long trusted as a stable cryopreservation for a wide variety of cells including mouse ES cells. Therefore, we improved the conventional slow-freezing cryopreservation using 10% DMSO by simply methods. This method requires neither specific equipment nor reagents and results in the high levels of growing cell numbers and undifferentiation states. Because the conventional slow-freezing cryopreservation is quite familiar, this simple method would gain widespread acceptance.

Poster Board Number: T-2316

THE HISTONE DEMETHYLASE UTX REGULATE CELL ADHESIN DURING DIFFERENTIATION OF HUMAN EMBRYONAL CARCINOMA STEM CELLS INTO NEURON

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JmjC domain-containing UTX and Jmjd3 are a histone H3K27 demethylase and demethylate trimethylated lys27 on H3. H3K27me3 is a chromatin modification that is repressed gene expression and is key factor for cell fate regulation. To determine the global gene expression changes after neural differentiation with reduction of similar enzyme either of UTX or JMJD3, we employed a DNA microarray analyses to analyze the differences between the gene expression patterns of among embryonic carcinoma cells transfected by lentiviral vectors with amiUTX (artificial miRNA targeting the UTX), amiJMJD3 (artificial miRNA targeting the JMJD3), or amiNC (normal control), respectively. After neural differentiation (for 4 weeks), differentiated cells were confirmed by immunocytochemistry. Both of amiNC and amiUTX transfected cells were differentiated into neuronal cells with typical neuronal markers such as β-tubulin type III. We observed that cells transfected amiUTX increases NCAM-mediated adhesion during neural differentiation. Both of UTX and Jmjd3 are a histone H3K27me3 demethylase, however they regulate different target genes during neural differentiation. Reduction of UTX gene expression promotes cell adhesion and differentiation human embryonal carcinoma stem cells into neuron.

Poster Board Number: T-2317

CULTURE OF STEM CELL-RELATED TELOCYTES ISOLATED FROM RAT ENDOMETRIAL TISSUE

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Introduction: Telocytes are c-Kit+ interstitial cells with long prolongations that extend from the cell body called telopodes. These prolongations and can reach exceptional lengths up to hundreds of μm and form attachments to a variety of cells. Although telocytes display electrical activity and have been observed to form prolongations that attach to myocytes, they are dissimilar from pacemaker Interstitial cells of Cajal. In cardiac tissue, telocytes potentially have a role in nursing and guiding stem cell maturation and trafficking via cell-to-cell contacts along telopoles that span from stem cell niches to functional areas of tissue. Here, we studied telocytes in the uterus using a rodent model. Materials and Methods: Use of animals was approved by the University Health Network Animal Care Committee. Adult, virgin female Wistar rats (n=5) were sacrificed and uteri were dissected. The parametrial connective tissue surrounding and serosa was removed. Uterine horns were cut longitudinally, rinsed with PBS and enzymatically digested using collagenase and trypsin. Released cells were washed with FBS to stop the enzymatic reaction. Cell fractions were plated on T75 flasks in DMEM 10% FBS with 1% penicillin/streptomycin and cultured for up to 4 weeks. Cells were fixed for 5 min using 100% methanol and immunocytochemistry was performed using anti-vimentin and anti-connexin 43. Staining was visualized with secondary Alexa 488 and Alexa 568 antibodies. Cultures were also stained with routine Giemsa staining. Cultures were photographed using a Nikon Ti Eclipse microscope. Results: Telocyte-like cells were not detected in endometrial luminal epithelial or endometrial glandular epithelial

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cell enriched fractions. Within 24 hrs in culture, cells in endometrial stromal cell enriched fractions were found with short or very long prolongations. Long prolongations spanned up to hundreds of µm in length. These prolongations often ended connecting to stromal cells on either end. These terminal connections often were made not to the nearest stromal cell, but to distant cells, with the prolongations sometimes extending over other stromal cells in between. Telopode forming cells were commonly found connecting two colony forming units (CFU) of stromal cells and in rare cases telopodes extended in parallel direction. Conclusions: Previous studies have shown that telocytes are found in a variety of tissues. Here, we show telocyte-like cells are also present in the uterine endometrium. Our findings suggest telocytes may have roles in cell-to-cell communication over short and long distances within the endometrium and may play a role in endometrial stem-cell interactions.

Poster Board Number: T-2318

FINDING THE HOME OF HUMAN TROPHOBLAST PROGENITORS

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Little is known about the progenitor cells that give rise to human trophoblast populations. Using immunohistochemistry on first trimester placental villi, we investigated where the niches for these trophoblast progenitors are located based on known mouse trophoblast stem cell markers. We found that staining for TCFAP2C (also known as AP-2γ) is concentrated at the base of cytotrophoblast cell columns. This correlates well with the staining pattern for Ki-67. Interestingly, there was unexplained widespread staining by anti-phosphorylated histone H3 in villous and extravillous trophoblast. GATA3 is expressed only in extravillous trophoblast and not in villous cytotrophoblast. Estrogen-related receptor beta (ERRβ) is expressed in most cells within the placenta. We provide further supporting evidence that there is a niche at the base of trophoblast cell columns where a progenitor population resides and where the cells are TCFAP2C, GATA3 and ERRβ positive.

Poster Board Number: T-2319

EFFECT OF CYCLOPHOSPHAMIDE ON COLONY-FORMING PROGENITOR CELLS OF STROMAL FIBROBLASTS

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Hemopoiesis occurs in organs that provide the conditions for hemopoietic and stromal cell interaction. These conditions are formed by specific microenvironment that represents the system of cellular and humoral factors being essential for driving, maintaining and differentiating of stem hemopoietic cell and its descendents. Stromal fibroblasts are responsible for specific microenvironment transfer. Only these cells following reverse transplantation *in vivo* are known to form hemopoietic loci typical of the initial organ (Fridenstein A.J. et al., 1974). Multiple investigations revealed that properties and state of microenvironment could considerably change under different effects (irradiation, mechanical injuries, hemorrhages, and chemical factor action), that could be judged by the variation in the number of progenitor cells of stromal fibroblasts in bone marrow, and by the dynamics of their ability to form colonies. The aim of this work was the analysis of the alteration in the number of colony-forming progenitor cells of stromal fibroblasts in bone marrow and their clonogenic potential in response to the effect of cyclophosphamide, the preparation that is applied in clinical practice in therapy of many diseases, and particularly in anti-tumor therapy. There was used the method of cloning the stromal progenitor cells of bone marrow from male mice of CBA strain using monolayer cultures with a-MEM medium and 20% calf serum under specific gaseous environment and temperature; following the feeder adhesion the irradiated hemopoietic cells of bone marrow from guinea pigs were added. Mice were intraperitoneally pre-injected with cyclophosphamide in doses of 200 mg/kg or 275 mg/kg, after which bone marrow cells from experimental animals were explanted in cultures at different terms from 1 to 13 days since injection of preparation. Following fixation and staining after 10-12 d-cultivation the number of colonies was calculated in cultures when colonies comprised no less than 50 cells. Under the effect of cyclophosphamide the content of stromal clonogenic cells (CFU-F) in red bone marrow undergoes significant variation. After 12 h since injection of preparation their number reduces to 4% of the control value with dose of 200 mg/kg and up to 5% with dose of 275 mg/kg. In a day the amount of CFU-F increases by 12-fold with dose of 200 mg/kg and by 5-fold with dose of 275 mg/kg. CFU-F content is subjected to a secondary decrease on the 2nd day following the cyclophosphamide injection (up to 12% of control values with dose of 200 mg/kg and up to 3% with dose of 275 mg/kg). Only 7-9 days after the agent introduction the amount of CFU-F content in bone marrow approaches relatively constant level. As a whole, the dynamics of variation in CFU-F content in bone marrow after the cyclophosphamide injection is similar to the variation pattern in the number of these cells after the irradiation with low doses. However the cytostatic possesses more attenuated action on CFU-F as compared with irradiation, as cell regeneration proceeds faster after the cyclophosphamide injection. This work was supported by RFBR grant 11-04-96037r_ural_a and administrative body of Perm Region.

Poster Board Number: T-2320

REGISTRATION OF HUMAN EMBRYONIC STEM CELL LINES IN KOREA

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In an effort to increase the credibility of human embryonic stem cell lines established in Korea, an obligatory registration was introduced into the Bioethics and Safety Act 2008 and started from January 1, 2010. A total of 79 domestic human embryonic stem cell (hESC) lines were submitted to Korea Centers for Disease Control and Prevention (KCDC) for the registration until 2011. The submitted lines were characterized in the respects of DNA fingerprint, chromosome stability, expression of stem cell markers, and contamination of mycoplasma by KCDC. The characterization data and ethical aspects such as informed consents for donation of surplus embryos were reviewed by an advisory review board for stem cell registry. 59 hESC lines were registered until 2011. This registration has completed an oversight system for the embryo research by registering the products of licensed embryo research projects. The information about hESC lines is available at Korea Stem Cell Registry (kscr.nih.go.kr).

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Poster Board Number: T-2322

CHARACTERISTICS OF MULTIPOTENT STEM CELLS DERIVED FROM THE EPIBLAST AND ECTODERM OF POSTIMPLANTATION MOUSE EMBRYOS

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Among the self-renewing cells that express molecular markers of pluripotency, the epiblast stem cells (EpiSCs) have been derived from epiblast of postimplantation mouse embryo at stages prior to and during germ layer formation. It is not known at which advanced developmental stage that EpiSCs-like cells can still be derived from the epiblast or the ectoderm of the embryo, and if the characteristics of EpiSCs derived from embryos of different developmental stage may reflect those of the tissue of origin. In this study, epiblast or ectoderm from a wide range of developmental stages defined by stringent morphological criteria were tested in an identical culture condition for the ability to generate EpiSCs. Over 40 EpiSCs lines were derived from epiblast at two pre-gastrula stages: cavity and pre-streak, and at several gastrulation stages: early-streak, mid-streak, mid to late-streak, late-streak and at stages as advanced as from ectoderm of early bud and as neural plate stage. EpiSCs derived from all stages display similar morphology and expressed the pluripotency markers Oct4, Sox2 and Nanog. Transcriptome analysis revealed that all these lines show gene expression profiles comparable to established EpiSCs but different from those of embryonic stem cells. EpiSCs lines derived from similarly staged embryos are transcriptionally more closely related to each other than with those from other developmental stages. The lineage potential of the EpiSCs was tested by teratoma formation assay and *in vitro* differentiation as embryoid bodies (EBs). EpiSCs lines produced teratomas containing derivatives of all three germ layers, with a preponderance of neural cells and a minor representation of endoderm and mesoderm derived tissues. When grown as EBs in serum-containing medium, EpiSCs from later stages of development down-regulate pluripotency markers earlier than EpiSCs derived from cavity stage embryo and expeditiously express the differentiation markers Sox1, Brachyury, and Mixl1. EpiSCs from advanced embryo therefore are more poised for undertaking germ layer differentiation. Ongoing analysis comparing the transcriptome of EpiSCs and dissected epiblasts of corresponding stages will reveal the relationship between EpiSCs and the epiblast from which they were derived and shed further light on the mechanisms of pluripotency maintenance and lineage commitment.

Poster Board Number: T-2323

GENETIC AND EPIGENETIC INSTABILITIES OF SEX CHROMOSOMES IN PLURIPOTENT STEM CELLS

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Our aim is to study the genetic and epigenetic mechanism that regulate X and Y chromosome in human and mouse pluripotent stem cells. In mammalian females, X chromosome inactivation is a process in which one of the two X chromosomes is silenced, following Xist expression. Unlike somatic cells, mouse embryonic stem cells (mESCs) do not express Xist, and harbor two active X chromosomes. Mouse induced pluripotent stem cells (miPSCs) also show two active X chromosome, implying that X is reactivating during the reprogramming process. However, analysis of X inactivation in human pluripotent stem cells, mainly based on Xist expression, was not conclusive. Here, we studied X-inactivation in human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) by meta-analysis of the expression of the entire set of genes on the X chromosome in female pluripotent cell lines. Thus, we could divide the human cell lines into three categories: lines with no X-inactivation, lines with full X-inactivation, and lines with partial X-inactivation. The partial inactivation of the X chromosome always involved the middle of the chromosome, surrounding the Xist transcription site. The status of XCI in some of the cell lines was validated by either allelic specific expression or DNA methylation analysis. Based on our analysis we propose a model for the dynamics of XCI in pluripotent cells. In this model, pluripotent stem cells show three interchangeable states of XCI. Thus, while mouse pluripotent cells show a stable state of two active X chromosomes, the variations in human XCI might be created during the reprogramming process, and/or result from epigenetic changes in culture. We next decided to examine genetic changes in sex chromosomes. We have recently developed a tool based on gene expression arrays that enable a reliable analysis of aneuploidy in autosomes. However, evaluating the stability of sex chromosomes poses difficulties due to the small size of the Y chromosome and the variability in X inactivation. We have thus adjusted the methodology to rely on the expression of only few genes, and then analyzed the stability of X and Y chromosomes in pluripotent stem cells. Surprisingly, whereas Y chromosome loss was identified in 15-30% of mouse male cell lines, no evidence for this phenomenon was found in human pluripotent cells. Furthermore, by comparing aberrant and normal cell lines from the two species, we identified candidate genes which may contribute selective advantage to the aberrant cell lines. Finally, we use these genes to study the connection between the different processes of genetic and epigenetic stability.

Poster Board Number: T-2324

COMBINATION OF FEEDER CELLS GENERATES PROPER ENVIRONMENTAL NICHE FOR MOUSE HEMATOPOIETIC STEM CELLS

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A variety of in vitro culture conditions have now been described that permit expansion of hematopoietic stem cells(HSC). Yet, so far it has not been possible to expand HSCs *ex vivo* without losing their stemness properties. The ability of stem cells to self-renew and to differentiate into different types of mature cells depends on both their intrinsic genetic programs and external control from the special microenvironment or niche where they reside. In adult bone marrow, niche of hematopoietic stem cells is composed of many different cell type, including osteoblasts, adipocyte, stromal cells, and vascular endothelium cells. Osteoblasts are thought to provide factors that are crucial for the maintenance of the quiescent status of HSC, whereas BM stromal stem cell/fibroblasts produce a large variety cytokines that promote proliferation. In order to study how these signals are integrated and whether the balance between quiescent, self-renewal and differentiation can be achieved in long-term culture, we developed a special feeder system for HSC culture by mixing various amounts of genetically modified stromal cells with mesenchymal stem cells (MSC) before they were induced to form bone. We found that the system using stromal cells expressing BMP antagonist, noggin together with MSC, could reproducibly generate a pattern of thin osteoblast strips surround by stromal cells on the tissue culture disc. Compared to undifferentiated MSC, this feeder system (NSMB) produced higher level of Jagged-1, angiopoietin-1, wnt-3a, various extra cellular matrix proteins, as

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well as BMP2, 4 and TGF-β. When lin-sca1+c-kit+ mouse HSC were cultured on NSMB they generated both cobble stone area and a large amount of differentiated cells. After one month in culture, cells cultured on NSMB generated 4 times higher number of LTC-IC than those grown on MSC. Interestingly, LSK HSC cultured on NSMB produced significant higher number of colony forming unit granulocyte, erythrocyte, macrophage and megakaryocyte (CFU-GEMM) than other feeders. Our results suggested that this feeder system might be able to promote cells proliferation as well as HSCs maintenance.

Poster Board Number: T-2325

THE ASYMMETRIC DIVISION OF DAMAGED PROTEINS IS STEM CELL-TYPE DEPENDENT

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An asymmetric division of damaged proteins during mitosis has been linked to the protection of one cell from aging in yeast and bacteria. Recent evidence suggests that stem cells may employ a similar mechanism; however, there is no in vivo evidence suggesting this occurs in healthy adult stem cells. We report that stem cells in the larval (neuroblast, NB) as well as adult (female germline, GSC and intestinal stem cell, ISC) *Drosophila* asymmetrically segregate damaged proteins, such as the difficult to degrade and age-associated 2,4-hydroxynonenal (HNE), and we propose that the stem cell niche is involved in this segregation. Surprisingly, we found that only the ISC protects itself by segregating HNE to differentiating progeny while the NB and GSCs retain HNE during asymmetric division compared to their progeny. The ISC has a high level of delta, the Notch ligand, and the differentiation of its daughter enteroblast, which expresses high levels of Notch, depends on the interaction between the stem cell and enteroblast. In a temperature sensitive Notch mutant (NTS1), clusters of delta-positive cells can arise when Notch signaling is lost between the ISC and enteroblast and only proliferation of ISC/enteroblast-like cells is seen. We found that in these clusters of poorly differentiated delta-positive cells, there was a variation in HNE intensities between cells. This suggests that the mechanism of HNE segregation remains intact when differentiation is disrupted. DE-Cadherin, involved in anchoring stem cells to their niche, colocalizes with HNE within the asymmetrically dividing cells of each *Drosophila* stem cell model. Furthermore, mechanical disruption of the stem cell niche and mutation of the extracellular domain of DE-cadherin abolishes HNE asymmetry in neuroblasts. Even when stem cell fate is disrupted in NTS1 intestinal stem cells, HNE remains colocalized with DE-Cadherin within cells of delta-positive cell clusters. We propose that the anchorage of damaged proteins to DE-cadherin is one mechanism that allows stem cells to segregate damaged proteins. Furthermore, we predict that this segregation will always be to the cell that has the shortest functional lifespan, as the divisions in all three stem cell populations were asymmetric with respect to cell fate, lifespan, mitotic activity and HNE, but in each case it was the cell with the shortest functional lifespan, regardless of mitotic activity or cell fate, that received the majority of damaged proteins during division.

Poster Board Number: T-2326

AMINO ACID DEPLETION CONTRIBUTES TO THE IMMUNE PRIVILEGE OF MOUSE EMBRYONIC STEM CELLS AND TISSUES DIFFERENTIATED FROM THEM

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We have shown previously that tissues derived from mouse embryonic stem cells (mESC) display a fragile form of immune privilege that secures their indefinite survival across a minor histocompatibility barrier. This form of immune privilege depends on the polarisation of infiltrating T cells towards an induced regulatory phenotype (iT_{reg}). Given increasing evidence that depletion of essential amino acids (EAA) plays an important role in conferring on otherwise immunogenic tissues protection from immune responses, we investigated whether amino acid catabolism might determine the immunological properties of ESC. We have shown that a number of candidate enzymes responsible for depleting EAAs are expressed at elevated levels by mESC and are responsible for the active depletion of lysine, threonine and valine from the culture medium. Furthermore, medium conditioned by mESC inhibited T cell proliferation and biased the polarisation of $CD4^+$ T cells towards a $FoxP3^+$ iT_{reg} phenotype *in vitro*, similar to control medium in which lysine, threonine and valine were limiting. In order to investigate whether depletion of any one of these EAAs might confer immune privilege, we generated mESC deficient in the enzymatic function of cytosolic branched chain aminotransferase (Bcat-1), an enzyme responsible for the catabolism of branched chain amino acids including valine. Our results show that expression of Bcat-1 alone is not sufficient to confer immune privilege *in vitro* and *in vivo*, but likely works collaboratively with other enzymes to create a protective microenvironment. Indeed, we were able to show redundancy at the level of the cytokine milieu by demonstrating the active secretion of TGF-β by mESC, the neutralisation of which partially inhibited iT_{reg} induction. Our data therefore suggest that mESC employ multiple mechanisms to establish a form of acquired immune privilege, including the depletion of EAAs.

Poster Board Number: T-2327

OLIGODENDROCYTE PRECURSOR CELL DIFFERENTIATION INDUCING FACTORS AS A REGENERATIVE THERAPY FOR MULTIPLE SCLEROSIS

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Multiple sclerosis (MS) is an autoimmune disease characterized by demyelination of axons and subsequent neuronal dysfunction. MS is primarily mediated by T-lymphocytes, which trigger inflammatory processes targeting myelin-producing oligodendrocytes (OLs), causing lesions in the myelin sheath, eventually leading to impaired neuronal conduction. Modulating the pathological immune response is the primary focus of current therapeutic approaches for the treatment of MS. While these treatments are moderately effective in the early stages of MS, none have proven to modify the course of primary progressive or relapsing- remitting MS. In the central nervous system, a widespread proliferating population of nerve/glial antigen-2 (NG2), platelet-derived growth

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factor receptor (PDGFRα) positive adult stem cells, termed NG2-glia or oligodendrocyte precursor cells (OPCs), are the major source of newly formed mature OLs required for remyelination. Remission in MS is largely dependent upon OPCs migrating to sites of injury, and subsequently differentiating to a mature cell fate capable of repair. However, even with immune suppression, progressive phases of MS are associated with inhibited differentiation of OPCs, wherein OPCs migrate to lesions in the spinal cord, but either fail to differentiate or form immature OLs that do not produce myelin. As such, the identification of drug-like small molecules that selectively induce differentiation of OPCs into mature myelin producing OLs at sites of demyelinated lesions may provide an effective alternative strategy of enhancing remyelination as a treatment for MS. We developed a High Throughput-High Content Imaging based screening platform with the expression of myelin basic protein (MBP) as a readout, to rapidly identify molecules that induce the differentiation of rat primary OPCs into functional myelinating OLs. This platform was used to screen large libraries of drug-like small molecules, natural products and other pharmacologically active agents, and led to the identification of several classes of novel compounds that induce robust differentiation of OPCs *in vitro*. Amongst these classes of effective compounds was BA404 (EC_{50} ~350 nM), which showed excellent brain exposure properties and *in vivo* safety profile. BA404 showed promising preliminary *in vivo* efficacy in pre-clinical animal models for MS. Evidence derived from *in vitro* and *in vivo* T-lymphocyte assays with BA404 indicates that the observed efficacy of this compound results from an enhancement of remyelination rather than immune suppression. Detailed *in vivo* characterization as well as mechanism of action studies with BA404 are currently in progress. Identified molecules from other classes, possessing suitable drug-like properties are also being evaluated using *in vitro* and *in vivo* models for MS and could serve as potential candidates for a regenerative therapy for MS. In addition, pharmacological and mechanistic studies using these novel regulators of OPC differentiation could serve to elucidate new pathways and targets regulating the remyelination process. These molecules that harness endogenous adult stem cells (OPCs) to effectively regenerate the myelin sheath *in vivo*, could have a significant impact on the development of clinical combination regimens with existing immuno-modulatory drugs for the treatment of MS and other demyelination related diseases.

Poster Board Number: T-2328

CHARACTERIZATION OF RAT CARTILAGE-DERIVED PROGENITOR CELLS ISOLATED AT DIFFERENT DEVELOPMENTAL STAGES.

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Introduction: Fetal cartilage-derived progenitor cells (FCPCs) are a promising cell source for cartilage tissue engineering because they have high abilities for both cell proliferation and chondrogenesis. However, FCPCs have limitations that they are not clearly identified and characterized yet particularly depending on the developmental stages. This study focused on comparing FCPCs at gestation ages of 14 (mesenchymal cell condensation stage), 16 (chondrocyte maturation and hypertrophy stage) and 20 (separation of cartilage growth regions, vascular invasion, and initiation of both cortical and trabecular bone stage) days in terms of their stemness and chondrogenic potential. Materials and methods: The cells isolated from rat fetal cartilage in gestation ages 14, 16, and 20 days were compared with adult rat MSCs and chondrocytes. Fetal cartilages isolated were first rinsed with PBS, and muscles are trimmed and minced in small pieces. And then digested with 0.1% collagenase in Dulbecco's modified Eagle's medium containing penicillin and streptomycin at 37°C for 16hr. Cells were than cultured in monolayer until passage 2, while doubling time of cells was measured. To evaluate multi-lineage differentiation ability of FCPCs, cells were differentiated into chondrogenic, adipogenic and osteogenic lineages for 14 and 21 days in vitro and evaluate by Safranin O, Oil Red O, Alizarin Red S stainings, respectively. Then, FCPCs (5,000,000cells/50ul) were injected into the back of nude mice (female, 6-week old) and analyzed for cartilage tissue formation *in vivo* after 14, 21 days by gross image and safranin O staining. Results and conclusion: In the in vitro study, rat FCPCs in post-gestational age of 20 days showed highest multipotentent differentiation ability. Similarly they also showed superior chondrogenic potential in the *in vivo* study. GA16 and GA 20 cells had a shorter doubling time than GA14. Our data suggested that regeneration capacity and stemness of FCPCs were dependent on developmental stage and FCPCs at gestational age of 20 days could be useful for cartilage regeneration.

Poster Board Number: T-2329

REPLICATIVE SENESCENCE OF HMSC IN CULTURE IS ASSOCIATED WITH ANEUPLOIDY AND THE DEREGULATION OF PLOIDY CONTROLLING GENES

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Human mesenchymal stem cells (hMSC) are being successfully used in cell therapy clinical trials for the treatment of a range of pathological conditions, including graft versus host disease (GVHD), bone and autoimmune defects, complex fistula and myocardial infarction. The majority of these clinical trials use hMSC that have been expanded *in vitro* for 8-10 weeks under pro-oxidative "standard" cell culture conditions. These conditions could have negative effects over genetic stability and promote mutations and chromosomal abnormalities. Our FISH (Fluorescence *in situ* hybridization) analysis shows that aneuploidy is not unusual phenomenon in conventional cultures of hMSC and that it progressively increases with the passages. We further demonstrate that senescence is linked to transcriptional deregulation of a set of genes that have been previously implicated in cancer and ploidy control. The immortalization of hMSC by overexpression of hTERT, reversed the deregulation of these ploidy control genes maintains the basal levels of ploidy even during long-term culture. We also found that ectopic hTERT expression preserves ploidy levels through its canonical function of telomere elongation and by reducing the levels of oxidative stress in these cells. We propose that the high levels of aneuploidy and deregulation of these genes would be relevant biomarkers of senescence in standard cultures of hMSC.

Poster Board Number: T-2330

RED AND NEAR INFRARED LIGHT IRRADIATION INCREASE PROLIFERATION AND MIGRATION OF RAT BONE MARROW MESENCHYMAL STEM CELLS

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Low level light irradiation (LLLI) was found to exert positive effects on various cells *in vitro*. The aim of this study was to investigate the effect of LLLI on proliferation and migration of rat bone marrow mesenchymal stem cells (rbMSCs). Light irradiation was applied

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at the energy density of 4 J/cm^2 using red (630 nm, 15 mW/cm^2) and near infrared (NIR, 850 nm, 10 mW/cm^2) light emitting diodes (LEDs). The number of rbMSCs with NIR illumination increased 27 %, whereas the number of cells with red light irradiation increased 5.6 %, compared to non-irradiated control group at the fourth day after receiving light exposure. Wound healing assay showed both red and NIR light irradiation increased cell mobility. Red and NIR light enhanced transmembrane migration of rbMSCs up to 292.9 % and 263.6 % accordingly. The transmembrane migration abilities of red and NIR light treated cells were decreased to 175.8 % and 182.2 % as compared to control cells upon addition of NF-κB inhibitor-quinazolinediamine, respectively. The expression of tissue inhibitor of metalloprotease (TIMP) -1 and -2 genes was reduced immediately after red light irradiation. The secretion of MMP-2 and MMP-9 was facilitated and pFAK expression was elevated after red and NIR LLLI. The study demonstrated that red and NIR LLLI increased rbMSCs proliferation, migration and identified the phosphorylation of FAK as a critical step for the elevated cell migration upon LLLI.

Poster Board Number: T-2331

TEMPORAL OSCILLATION IN PROTEIN EXPRESSIONS GENERATES ROBUST DIFFERENTIATION DYNAMICS OF STEM CELLS

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A systems-level understanding of cell differentiation from multipotent stem cells to committed cell types is essential to the advance in developmental biology, and is also an urgent topic for potential medical applications. Some of the basic questions concerning such systems-level understanding include: What characteristics in a cellular state distinguish multi-potent stem cells from differentiated cells? How are developmental processes robust to molecular noise in spite of their complexity? Following the progress in the analysis of the dynamics of gene expression and the structure of gene regulatory networks, the time is ripe to answer the above questions to unveil nature of differentiation from stem cells. In this study, using a dynamical system modeling cell approach, we performed simulations of the developmental process using all possible topologies of gene regulatory networks with a few genes, and screened those that could generate cell type diversity through cell-cell interactions. We found that stem cells that both proliferated and differentiated always exhibited oscillatory expression dynamics, and the differentiation frequency of such stem cells was regulated, resulting in a robust number distribution. Moreover, we uncovered the common regulatory motifs for stem cell differentiation, in which a combination of regulatory motifs that generated oscillatory expression dynamics and stabilized distinct cellular states played an essential role. Based on the result of computer simulations, we propose a hypothesis for the mechanism of stem cell differentiation and its regulation to maintain robust developmental processes, in which the expression levels of some genes in multipotent stem cells exhibit temporal oscillation, and itinerate over several sub-states. As development progresses, each of these quasi-stable sub-states is modified and stabilized, leading to differentiated cell types. This temporal oscillation in expressions introduces variety in state changes and maintains regulated differentiation from stem cells, resulting in a robust number distribution of cells of different types. Importantly, this hypothesis can explain the roles and mechanism of the recently observed dynamic heterogeneity [1-4] and oscillatory behavior [5] in cellular states of stem cells, and it can predict the regulatory motifs responsible for the dynamic differentiation process. Based on the comparison between experimental observations and the results of computer simulations, we make several predictions on a network structure and cellular state to provide stemness. These discussions promote a system-level understanding of multicellular development and provide a basis for clinical application of stem cells. [1] Toyooka Y, et al. (2008) Identification and characterization of subpopulations in undifferentiated ES cell culture. Development 135(5): 909-918. [2] Chambers I, et al. (2007) Nanog safeguards pluripotency and mediates germline development. Nature 450(7173): 1230-1234. [3] Hayashi K, et al. (2008) Dynamic equilibrium and heterogeneity of mouse pluripotent stem cells with distinct functional and epigenetic states. Cell Stem Cell 3: 391-401. [4] Chang HH, et al. (2008) Transcriptome-wide noise controls lineage choice in mammalian progenitor cells. Nature 453: 544-548. [5] Kobayashi T, et al. (2009) The cyclic gene Hes1 contributes to diverse differentiation responses of embryonic stem cells. Genes and Dev 23(16): 1870-1875.

Poster Board Number: T-2332

GENES INVOLVED IN PROLIFERATION OF PLANARIAN PLURIPOTENT STEM CELLS

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Planarians have remarkable regenerative ability. Planarians can regenerate their whole functional body from a tiny fragment within one week. This regenerative ability of planarians absolutely depends on a unique population of pluripotent stem cells called neoblasts. Neoblasts are the only cell population possessing ability to proliferate and to differentiate into all types of cells in planarians. Therefore, neoblasts play an important role in regeneration after artificial amputation or fission and tissue homeostasis. Fission is the means by which asexual reproduction normally occurs in planarians: it consists of division into two fragments dependent on body growth. Fission occurs in the pre- or post- pharyngeal region when planarians grow to a sufficient size (about 8~9 mm) in rich nutrient conditions after feeding. After feeding, transient acceleration of neoblast proliferation is observed. This temporary increase of proliferation of the neoblasts is called a mitotic burst, and a mitotic burst is also induced during regeneration. After this mitotic burst, neoblast proliferation returns to the normal rate in one week but the molecular mechanism which causes the increase and restoration of neoblast proliferation still remains unclear. In order to understand the mechanism that controls the acceleration of neoblast proliferation after feeding and amputation, we tested some candidate neoblast-specific genes obtained by the HiCEP method. One of the genes, *DjP2X-A* (a Dugesia japonica ionotropic ATP receptor, a P2X homologue), was expressed in about half of the neoblasts. Intriguingly, RNAi of *DjP2X-A* showed that RNAi planarians enhanced their fission frequency at a threshold body size in the nutrient-rich condition. We found that expression of *DjP2X-A* was negatively correlated with mitotic burst after feeding, suggesting that *DjP2X-A* is involved in restoring the neoblast proliferation state after feeding. Indeed, we revealed that mitotic burst after feeding was increased by RNAi of *DjP2X-A*. A similar result was obtained in *Plac8* (Placenta specific gene8 homolog ; a candidate that was also obtained by the HiCEP method) knock-down planarians. RNAi of *Plac8* enhanced fission frequency at a threshold body size, like RNAi of *DjP2X-A*. In addition to this, we could observe similar results in expression dynamics of *Plac8* after feeding with that of *DjP2X-A*, suggesting that *Plac8* has similar function to *DjP2X-A* in modulating neoblast proliferation after feeding. In planarians regenerating after artificial amputation, the expressions of *DjP2X-A* and *Plac8* disappeared from the neoblasts located in the vicinity of the wound where mitotic burst of

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the neoblasts was induced. These observations and results indicate that multiple factors regulate the rate of stem cell proliferation in both homeostasis and regeneration in planarians. Here we show the function of *DjP2X-A* and *Plac8* in proliferation of the neoblasts and discuss the relation between these genes and growth and regeneration.

Poster Board Number: T-2333

IDENTIFICATION OF CRITICAL MIRNAS INVOLVED IN HUMAN MSC MOTILITY AND CELL GROWTH BY RNA-SEQ TECHNOLOGY

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Mesenchymal stem cells (MSCs) found in bone marrow (BM-MSCs) are an attractive source in regenerative medicine. MSCs can also be obtained from other postnatal, perinatal or fetal tissues, yet stem cells isolated from different origins express distinct biological activities, such as differentiation abilities. We recently showed that MSCs from Wharton's jelly of umbilical cord (WJ-MSCs) are more primitive and similar to embryonic stem cells, while BM-MSCs were more efficient in osteogenic and adipogenic differentiation. On the other hand, WJ-MSCs expressed less genes involved in immune response (such as CXCL12 and HLADRA) and have a higher immunosuppressive effect over allogenic macrophages, indicating WJ-MSCs being a better choice in allogeneic transplantation (Hsieh et al., 2010). In this following study we explored other biological differences between these 2 MSCs and applied RNA-seq technology for understanding the underlying mechanisms. We found that BM-MSCs possess higher motility than WJ-MSCs, while WJ-MSCs proliferated better. These differences may be the outcome of higher NF-κB activity in WJ-MSCs than in BM-MSCs. To understand the molecular mechanisms of these phenotypes, we compared the transcriptome profiles between BM- and WJ-MSCs using miRNA-seq. We identified hundreds of miRNAs differential expressed in BM-MSCs and WJ-MSCs, and miR-146a contributes in MSC motility and proliferation ability through targeting CXCL12 and SIKE1, respectively. Knockdown miR-146a down-regulated NF-κB activity, and NF-κB activity can be partially recovered by double knockdown SIKE1 in WJ-MSCs. Novel miRNAs which have never been deposited in databases were also found from our RNA-seq data and could be verified by RT-PCR and RNA-IP with AGO2 antibody. Our results provide the different biological properties of two kinds of MSCs from different sources, as well as the underlying regulatory miRNAs and genetic networks, thereby providing a better basis for cell-based therapy and the following mechanistic studies on MSCs biology.

Poster Board Number: T-2334

CARDIAC MITOCHONDRIAL INTEGRITY REGULATED BY CR6 INTERACTING FACOR 1

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Background and Objectives: The major cause of metabolic syndrome and diabetes is reduced cellular performances in fuel metabolism, but the underlying pathways and mechanisms are not completely understood. Dysregulation of energy homeostasis can lead to metabolic disturbances and it predispose diabetes, cardiovascular disease, aging and cancer. CR6-interacting factor(CRIF1) contacting colied-coil domain that is required for both genomic stability and mitochondrial integrity. We performed this study to determine the role of CRIF1 on the mice hearts. Methods and results : CRIF1-deficient mouse was embryonic lethal, we made heart specific CRIF1-deficient mouse using Cre-loxP system. We made thoracotomy and directly injected adeno-Cre virus into the heart of CRIF1 loxP mice. Betagal virus was used as a control. Serial echocardiography showed decreased left ventricular ejection fraction and fractional shortening in the CRIF1-deficient mice at four and seven weeks later compared to wild type mice ($p<0.05$). H&E showed increased myocardial inflammation in the CRIF1-deficient mice. TUNEL staining and LC3 staining showed increased apoptosis and autophage in CRIF1-deficient mice compared with wild type($p<0.01$). Electromicopy revealed that the mitochondria in CRIF1-deficient cardiomyocytes showed abnormal morphogenesis. For example, the cells showed excessively fragmented mitochondria and intracriatal swelling, thinning of myocardial fiber. The stability of mitochondrial complexes in CRIF1-deficient cells showed marked derangements. Conclusion: CRIF1 is required for maintenance of normal mitochondrial function and modulate apoptosis and autophage in heart.

Poster Board Number: T-2335

PLACENTA-DERIVED MULTIPOTENT CELLS (PDMCS) INDUCE FUNCTIONAL $CD4^+CD25^{HIGH}FOXP3^+$ T REGULATORY LYMPHOCYTES

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Recent advances in stem cell research have brought new hope for regenerative medicine through cell therapy. However, immunological rejection is a major concern unless transplantation is autologous, which would severely limit prevalent use of stem cell products. Interestingly, one population of adult stem cells (ASCs), the mesenchymal stem cells (MSCs), has been found to possess immunosuppressive properties which allow for allogenic use. However, these ASCs are rare, with numbers decreasing with age. Placenta-derived multipotent cells (PDMCs) share many properties with adult MSCs, including multilineage differentiation and immunomodulatory capacity. Additionally, these fetal-stage progenitors possess embryonic stem cell surface markers and are exponentially more proliferative than adult MSCs, making PDMCs ideal for clinical applications requiring large cell volumes. Since T regulatory cells (Tregs) are one of the most important immunomodulatory effectors of the immune system, we explored whether the suppressive effects of PDMCs are mediated by this population of lymphocytes. We found that in mixed-lymphocyte reactions in which PDMCs are added as 3rd party cells, leukocyte cell division and proliferation can be suppressed. Co-culture of PDMCs with stimulated allogeneic peripheral blood leukocytes (PBL) also resulted in a shift away from a Th1 axis to a Th2 axis. This was correlated with an increase in IL-10 secretion as well as numbers of $CD4^+CD25^{high}$ lymphocytes, or Tregs. The numbers of Tregs increased over time as a fraction of total lymphocytes, and this can be sustained out to 8 days. These PDMC-induced $CD4^+CD25^{high}$ Tregs are $Foxp3^+$, non-dividing, non-proliferating lymphocytes, and functionally are able to suppress stimulated lymphocyte proliferation in a dose-dependent fashion. These findings along with multilineage differentiation capacity, and the ease of isolation/expansion may render PDMCs as an attractive source for cell therapy.

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SALL1 HAS DUAL FUNCTIONS IN THE KIDNEY DEVELOPMENT.

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Objectives;
The kidney develops through the reciprocal interaction between the ureteric bud and the metanephric mesenchyme. The metanephric mesenchyme surrounding the ureteric bud, called the cap mesenchyme, is characterized by the elevated expression of the transcriptional factor, Six2 (Sine oculis homeobox homolog 2). The cap mesenchyme represents the self-renewing progenitors, which eventually differentiate into the nephron, the basic structural and functional unit of the kidney. In the mouse embryonic kidney, both the cap mesenchyme and differentiating cells express the transcriptional factor Sall1 (Sal-like 1). Sall1 is a protein which has multiple double-zinc finger motifs. In *Sall1*-null mice, the ureteric bud failed to invade the metanephric mesenchyme, resulting in kidney agenesis. In cultured cells, Sall1 binds to co-repressor proteins, nucleosome remodeling and deacetylase corepressor complex (NuRD), and represses gene expression. However the precise mechanism on how Sall1 regulates kidney development has not been clarified. Here we investigated the role of Sall1 in the mouse embryonic kidney. Methods; Results; To examine the requirement for Sall1 in the cap mesenchyme, we generated *Sall1* floxed mice and intercrossed these mice to *Six2-GFP-Cre* mice. *Six2-GFP-Cre:Sall1flox/flox* mouse embryos showed reduced kidney size. The embryonic kidneys also showed decreased Six2 expression and poor maintenance of the mesenchyme structure. These results indicate that Sall1 plays a critical role in the maintenance of nephron progenitors in the cap mesenchyme. To reveal the details of *Sall1*-mediated regulatory mechanism, we generated a *Flag-tagged Sall1* knock-in mouse line. Co-immunoprecipitation analysis using *Sall1-Flag* mice kidneys showed that Sall1 was bound not only to the NuRD complex components, HDAC2 and Mi2β, but also to the transcriptional co-activating proteins, CBP and p300. As Sall1 binds to these two types of transcriptional co-regulators which have opposite roles, Sall1 could function both as a repressor and as an activator in the kidney development. Conclusions;Sall1 is essential for the maintenance of the cap mesenchyme in the developing kidney. Our results also showed that Sall1 binds to an activator and a repressor complex. As Sall1 is expressed not only in the cap mesenchyme but also in differentiating cells, Sall1 could function differently in these two populations, thereby maintaining the immature nephron progenitors.

Poster Board Number: T-2337

PRINCIPLES & POLYCENTRICISM: DESIGNING CROSS-BORDER REGULATORY SYSTEMS FOR BIOTECHNOLOGY - THE CASE STUDY OF STEM CELL RESEARCH.

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The problem of how to regulate emerging biotechnologies is a permanent issue on the modern political agenda. The rate of scientific advancement in fields such as, synthetic biology, assisted reproduction, enhancement, genomics and stem cell research, is such that there is renewed interest in governance mechanisms. This paper critically assesses the regulatory fit between principles-based, polycentric regulatory regimes (one strand of the new governance movement within contemporary regulatory theory) and biotechnology, using stem cell research as a case study. (Although each area of biotechnology presents unique issues, I contend that there are regulatory concerns that cut across the entire field.) I ultimately advance an argument in favour of this regulatory approach to biotechnology, considering along the way, issues of legality and the interface between ethics and regulatory design. Biotechnology - specifically, stem cell research - is an international, often collaborative sector; scientific work travels and translates across borders. Equally importantly, the ethical issues provoked by stem cell research are universal. Although national governments may compete to capitalize on scientific innovation, a number of international research initiatives have arisen in the field of stem cell research. This paper looks specifically at the international dimension of regulation. I argue that given the competing interests of parties (governmental and non-governmental institutions), the inescapable ethical challenges this research presents, and the rate of scientific progress, a polycentric, principles-based regime is the most appropriate and sustainable form of regulation in this field. The great attraction principles based regulation (PBR) holds for the field of stem cell research - indeed all biotechnologies - is the flexibility it affords both the regulator and regulatee. Given the speed of scientific advance - which by far outstrips the speed of the legislative, litigation, or political process - flexibility is key to effective, on-going regulation of current research activity that is also ethically defensible. PBR as an adaptive mechanism will, I submit, go some way towards addressing the regulatory 'lag' that often occurs in traditional, command and control systems of regulating biotechnologies. Underlying international collaborations in stem cell research are a myriad motivations and tensions. For regulators, balancing these interests whilst maintaining authoritative credibility is a challenge. I argue that a polycentric, principles-based regime is structurally secure form of regulation as it allows for the degree of pluralism often demanded in this field; principles afford flexibility, polycentricism disperses the task of regulation to the appropriate organization(s). Moreover, part of the strength of the argument in favour of international, polycentric, PBR is that the process of developing useable principles across regulators facilitates dialogue between different interest groups and regions/nations - which is arguably in itself, desirable.

Embryonic Stem Cell Pluripotency

Poster Board Number: T-3001

A HIGH QUALITY SERUM-FREE, FEEDER-FREE MEDIUM FOR HUMAN EMBRYONIC STEM CELLS (HESC) AND INDUCED PLURIPOTENT STEM CELLS (IPSC)

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The conventional method to culture human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) has been on irradiated murine embryonic fibroblasts (iMEFs) as feeder supported cultures or in iMEF conditioned medium. However, feeder supported cultures are time consuming, labor intensive and exhibit variability in cell performance. In addition, the morphology and cell growth in a feeder supported cultures are different.In order for researchers to enable effective pluripotent stem cell cultures, it is essential to have feeder free culture conditions with a culture medium that readily promotes adaptation of cells, promotes proliferation and maintains the pluripotent state of stem cells (PSCs) with

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consistency and reproducibility. Here, we report a new PSC feeder free medium that supports the expansion of hESCs and iPSCs with an ideal cellular morphology of compact colonies and high nuclear to cytoplasmic ratio. This feeder free culture medium has been further validated with different iPSC lines and shown to support robust proliferation for several passages with normal karyotype. This medium further supported the generation of iPSCs with the non-integrating CytoTune™ -iPS Sendai Reprogramming Kit under serum-free, feeder-free conditions on a Geltrex® coated matrix. The hESCs and iPSCs cultured or derived in this medium express normal pluripotency markers and differentiated to ectoderm, endoderm and mesodermal lineages via embryoid body generation. This culture medium enables feeder free cultures with ease and robustness.

Poster Board Number: T-3002

X CHROMOSOME INACTIVATION IN HUMAN EMBRYONIC STEM CELLS: ALL OR NOTHING!

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Introduction: Human embryonic stem cells (hESC) are often considered as an in-vitro model for human development and disease modelling, as well as a promising cell source for regenerative medicine, necessitating thorough characterization and monitoring of genetic and epigenetic (in)stability in hESC to ensure their quality. X chromosome inactivation (XCI), an example of epigenetic gene regulation, ensures dosage compensation of the X chromosome in female cells. After completion of XCI, the two X chromosomes in female cells can be distinguished by differential transcription of the XIST gene, DNA methylation, and epigenetic chromatin modifications. In this study we aimed to investigate the XCI pattern through analysis of DNA methylation in undifferentiated female VUB hESC lines at different time points during long-term culture. Materials and methods: hESC were cultured on inactivated mouse embryonic fibroblasts in standard SR-medium with bFGF addition, in 5% CO2 and atmospheric O2 concentrations. Undifferentiated hESC were collected at day 4 to 6 after passaging, using collagenase. DNA was extracted by phenol-chloroform. To determine if X-inactivation was present, whether it was random or skewed and to identify the parental origin of the inactivated X (whenever DNA samples of the donors are available e.g. lines derived from PGD embryos), we applied methylation-sensitive restriction by HpaII and PCR for the polymorphic aristaless related homeobox gene. Results: We examined a total of 17 hESC lines derived at the VUB, sixteen female lines and one male line as control. This male hESC line showed one PCR fragment for control DNA and absence of any fragment after restriction, proving the presence of an unmethylated locus on the X chromosome. Of the 16 female hESC lines, 4 lines (25%) displayed 2 active X chromosomes, even at later passages (up to P50). The remaining 12 lines all displayed XCI, however, with different patterns. Only two lines (13%) displayed the expected random XCI pattern at different passages, while nine lines (56%) showed a completely skewed XCI pattern. One line (6%) was not informative and although this line clearly shows XCI, we are not able to determine a random or skewed pattern. The lines with skewed XCI displayed this pattern already at early passages, e.g. P3 or P10. We were able to identify the active X chromosome as derived from the female donor in 4 lines, while in 4 other lines the active X was derived from the male donor. For one line, no DNA from the donors was available (VUB07). Discussion: It has already been shown that XCI in hESC is highly variable. This data set, however, is the first to show the extent of preferential inactivation of a specific X chromosome in hESC. In fact, only 13% of the hESC lines in this study displayed a random XCI pattern, while most lines had either no or skewed XCI. The presence and correct patterning of XCI in undifferentiated and certainly in differentiated hESC might be crucial for their applicability as in-vitro model for human development and even more as cell source for regenerative medicine. Therefore, in-depth studies on the effects of skewed XCI patterns on differentiation capacity and efficiency should be performed. Moreover, it would be of great interest to explore whether XCI skewing is a random event or whether it originates due to a selective advantage.

Poster Board Number: T-3003

ELABELA IN HUMAN EMBRYONIC STEM CELL PLURIPOTENCY AND DIFFERENTIATION.

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Based on expression profiling data we identified a novel gene, *ELABELA (ELA),* which is highly expressed in human embryonic stem cells (hESCs). To investigate the function of this gene we manipulated its expression levels in hESCs and derived over-expressing induced pluripotent stem cells (iPSCs) lines. Heterozygous *ELA* knock-out clones express lower levels of pluripotency markers than control hESCs and many markers of meso-endoderm are up-regulated. Cultures of iPSCs with elevated levels of *ELA* express higher levels of the pluripotency genes than controls, or *ELA* knock-out lines, and demonstrate increased proliferation. Although embryoid bodies generated from the *ELA* over-expressing iPSCs express genes representative of each cell lineage, extraembryonic markers are detected at a higher level than those in control iPSCs. These data suggest *ELA* plays a role in the regulation of pluripotency and differentiation in hESCs and in the balance between induction of definitive and extraembryonic endoderm.

Poster Board Number: T-3004

PROTEIN KINASE C INHIBITOR PROMOTES CONDENSED ORGANIZATION OF COLONIES FROM ENZYMATIC-DISSOCIATED SINGLE HUMAN EMBRYONIC STEM CELLS

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Human pluripotent stem cells (hPSCs) self-renew indefinitely as highly organized pluripotent colonies. Unlike mouse pluripotent stem cell colonies, human colonies form a uniform, flat epithelium-like monolayer. Interestingly, it has been reported that colony morphology is closely correlated with the maintenance of pluripotency. However, the molecular mechanisms that underlie human pluripotent colony formation and organization are poorly understood. In this study, using real-time imaging tools, we examine *in vitro* colony formation by enzymatically dissociated single hESCs under feeder-free conditions. We showed that colony formation consists of 4 stages: attachment, migration, colony formation (aggregation), and colony organization. Moreover, we found that blocking PKC signaling with isoenzyme-specific PKC inhibitors results in enhanced cell-cell interactions and plays an integral role in promoting the survival of hESCs in culture. Using various visualization methods, we identified conditions required for colony formation, and we suggested a promising new target for modulating hESC colony formation and organization. These results are likely to be useful for engineering hPSCs to further study the mechanisms involved in pluripotency.

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Poster Board Number: T-3005

ESSENTIAL ROLES FOR CULTURE CONDITIONS IN GENOMIC INSTABILITY AND CELL PHENOTYPE CHANGES OF HUMAN PLURIPOTENT STEM CELLS IN SHORT- AND MID-TERM *IN VITRO* CULTURE

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Human pluripotent stem cells (PSC) can self-renew in culture and have the potential to differentiate into all adult cell types. Therefore, PSC might provide *in vitro* models for human development and genetic disease and a source of cells for regenerative medicine. PSC can be obtained either from discarded embryos, i.e. embryonic stem (ES) cells, or by reprogramming differentiated cells into induced pluripotent stem (iPS) cells. However, recent evidence suggests that PSC might present genetic abnormalities that arise during cell culture or cell reprogramming and that are a serious concern for their use in *in vitro* or clinical applications. Here, we report that alterations of features of hESC could be induced even in short-term or mid-term *in vitro* culture, but firmly associated to the culture condition. In this report, three hESC lines (HD291, HD129 and HS306) at early passage were cultured in KO-SR, KO-DMEM and bFGF medium using one of two different passaging methods: (i) "single cell" passaging combined with ROCK-kinase inhibition or (ii) mechanical passaging ("cut and paste"). During the culture, the genome integrity and transcriptome of the cell lines was monitored by karyotyping, Affymetrix SNP6 DNA microarrays and mRNA sequencing. Morphology, cell proliferation, colonogenicity and expression of pluripotency markers were regularly monitored. Our data showed that HD291 cell line acquired quickly the copy number variation (CNV) changes under the "single cell" passaging, while the change of CNV in mechanical passaging was much less rapid. The same observation was also obtained for chromosome aberrations: HD291 cell line acquired a trisomy of chromosome 12 at passage 30 when using single cell passaging. The cells that were mechanically passaged showed a stable karyotype in this time frame. The genome alterations observed under single cell passaging were associated with cell phenotype changes such as an increased cell population growth rate, increased pluripotency marker expression, acquired colonogenicity etc., while no significant change was observed in the culture under mechanical passaging, even after 45 passages. Data acquisition for HD129 and HS306 is underway. Our data document for the first time the dynamic of the phenotypic and genetic changes in PSC during *in vitro* cell culture. In addition, we show that these changes are tightly correlated with the culture conditions such as single cell passaging. The data open the way to the understanding of the mechanism governing genetic alteration in stem cells in culture and may provide a framework to develop better PSC culture conditions.

Poster Board Number: T-3006

WNT-DEPENDNENT AND FGF/TGFB-INDEPENDENT HUMAN PLURIPOTENT STEM CELL RENEWAL

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Decision of self-renewal or differentiation in stem cells in vitro culture and *in vivo* is controlled by extrinsic factors such as signaling molecules. The extrinsic factors regulating human pluripotent stem cell (iPS cells or ES cells) self-renewal and early differentiation events seem to differ from mouse pluripotent stem cell and to date are incompletely understood, but activation of bFGF and TGFβ/activin/Nodal signaling form the cornerstone of most systems for human ES cell propagation. The Wnt/β-catenin signaling pathway plays an important role in mouse ES cell self-renewal in LIF-independent culture but it is dispensable in LIF-dependent culture. In human pluripotent stem cells, the role of Wnt/β-catenin signaling is still poorly understood and controversial because of the dichotomous behavior of Wnt/β-catenin signaling in proliferation and differentiation. While investigating small molecule chemical compounds that could segregate the dual role of Wnt signaling, we have identified a compound that could modulate Wnt/β-catenin signaling pathway and support Wnt-induced human ES cell self-renewal without affecting differentiation. Utilizing Wnt and the compound, we have developed a novel and simple chemically defined xeno-free culture system that allows for long-term expansion of human pluripotent stem cells without FGF or TGFβ supplementation. Cells in this culture can self-renew even with FGFR or ALK inhibitors. These culture conditions do not include xenobiotic supplements, serum, serum replacement or albumin. Using this culture system, we have shown that several human pluripotent cell lines maintained pluripotency (>20 passages) and a normal karyotype, and still retained the ability to differentiate into derivatives of all three germ layers. This Wnt-dependent and bFGF/TGFβ-independent culture system would provide a platform for complete replacement of growth factors with chemical compounds.

Poster Board Number: T-3007

THE *IN VITRO* SURVIVAL OF HUMAN MONOSOMIES AND TRISOMIES AS EMBRYONIC STEM CELLS

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Chromosomal aneuploidies are responsible for severe human genetic diseases. Aiming at creating models for such disorders, we have generated human embryonic stem cell (hESC) lines from preimplantation genetic screened (PGS) embryos. The overall analysis of more than 400 aneuploid PGS embryos showed similar occurrence of monosomy or trisomy in any specific chromosome. However, the generation of hESCs from these embryos revealed a clear bias against monosomies in autosomes. Moreover, only specific trisomies showed a high chance of survival as hESC lines, enabling us to present another categorization of human aneuploidies. The spectrum of aneuploidies in these hESC lines reflects the range of common embryonic chromosomal aberrations and significantly differs from the spectrum of aneuploid hESC lines generated by cell adaptation in culture. The aneuploid hESC lines represent an excellent model to study human chromosomal abnormalities especially in the early stages of development.

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Poster Board Number: T-3008

POTENTIAL ROLE FOR HYPOXIA INDUCIBLE FACTOR IN HPSC DIFFERENTIATION

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Human embryonic stem cells (hESCs) originate from the hypoxic microenvironment of the blastocyst inner cell mass. Hypoxia inducible factors (HIFs) constitute a family of labile transcription factors that regulate gene expression in response to hypoxia. HIFs are stabilized in low oxygen and are degraded by prolyl hydroxylation and subsequent proteolysis with increased intracellular oxygen levels. Recently, it was shown that HIF2a increases OCT 4 transcription to promote self-renewal in human pluripotent stem cells. Also, the loss of HIF1a, HIF2a and HIFa (ARNT) impair placentation, heart development, and endochondrial bone formation in knockout mice. These opposing roles for HIF activity generate a paradox in which HIFs seem to promote pluripotency but could also promote differentiation in distinct cellular contexts. To investigate this seeming paradox, we examined the role of HIF1a-mediated gene transactivation during early lineage non-specific differentiation under normoxic and hypoxic conditions. HIF1a was required for the induction of embryonic trigerm layer and trophoblast gene expression in both conditions. In contrast, ectopic HIF1a expression alone was insufficient to initiate spontaneous differentiation. Therefore, HIFs appear to have a dual role in hESC physiology- they prevent differentiation of self-renewing hESCs but can also promote hESC differentiation after its induction.

Poster Board Number: T-3009

COREGULATORS OF PRDM14 IN HUMAN EMBRYONIC STEM CELLS

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PRDM14, a PR domain-containing transcriptional regulator, was identified to be one of the major determinants of the human embryonic stem cells (ESC) identity in a genome wide RNAi screen. PRDM14 was shown to regulate Oct4 activity in the human ESC and therefore safeguards the pluripotency of the human ESC. On the other hand, previous studies have highlighted potential repressive role of PRDM14 in human ESC as the ectopic expression of PRDM14 represses the upregulation of lineage genes in embryoid bodies. To fully understand how PRDM14 can potentially play activating and repressive role in the human ESC, we perform biochemcal assays to identified potential PRDM14 interactors. We found that PRDM14 interacts with the core pluripotency factor Nanog in the human ESC and its binding to active genes are regulated by Nanog binding activity.

Poster Board Number: T-3010

INVESTIGATING THE EFFECTS OF EXTRACELLULAR MATRIX MOLECULES ON THE REGULATION OF HESC PLURIPOTENCY.

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Human embryonic stem cells are pluripotent cells that have indefinite replicative potential and ability to differentiate into derivatives of all three germ layers. HESCs are conventionally grown on mitotically inactivated mouse embryonic fibroblasts and some alternative feeder types of human origin have been used to culture hESCs. However, there are also supportive culture systems for hESCs lacking feeder cells, suggesting that extracellular matrix-stem cell interactions might contribute to the regulation of self-renewal or differentiation. Using defined feeder-free systems, we investigated the effects of receptor binding by blocking the integrin engagement to fibronectin and vitronectin. We have shown that hESCs express all the machinery for integrin signalling and blocking αVβ5 and β1 integrins led to a slight decrease in pluripotency marker expression. Moreover, selective chemical inhibiton of Src which is a receptor-independent signalling intermediate molecule, also resulted in a decrease in pluripotency marker expression. Thus this study further illuminates the role that ECM interactions play in the hESCs phenotype which has until recently been a neglected area of hESCs biology.

Poster Board Number: T-3011

MICRORNAS REGULATE P21WAF1/CIP1 PROTEIN EXPRESSION AND THE DNA DAMAGE RESPONSE IN HUMAN EMBRYONIC STEM CELLS (HESCS).

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Introduction: Studies of hESCs commonly describe the non-functional p53-p21 axis of the G1/S checkpoint pathway with subsequent relevance for DNA damage response (DDR). This is likely due to the absence of p21 protein in hESCs, which was repeatedly described, but never explained. Recently, microRNAs (miRNAs) were identified as crucial post-transcriptional regulators of gene expression and were directly linked with physiology of hESCs. Curiously, whether miRNAs play a role in the DDR and regulation of p53-p21 pathway in hESCs has not been studied thus far. Results: To address this question we used UVC irradiation (3 J/m2) as a DNA damaging agent in undifferentiated hESCs. The expression of miRNAs was analyzed at three different time points after UVC irradiation [0, 4, 8, and 16 hrs] using miRNA microarrays (LNA, EMBL). We identified 100 miRNAs (17.9%) whose expression increased (>1.5-fold) upon UVC irradiation (data were verified by qRT-PCR). Interestingly, 22% (22/100) of miRNAs upregulated upon UVC irradiation in hESCs are putative regulators of p21 mRNA as predicted by TargetScan (www.targetscan.org). Observed abundance of up-regulated miRNAs that are predicted to target p21 suggest their role in its regulation. We next tested the expression of p21 protein and other p53-downstream targets in UVC treated hESCs. Classical p53-downstream

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genes Bax, GADD45 and EGR1 were activated in hESCs upon DNA damage, but p21 protein was undetectable before or after UVC irradiation. Importantly, p21 mRNA was up-regulated in hESCs upon UVC (27-fold increase) indicating a major post-transcriptional regulation of p21 mRNA. Therefore, to directly address involvement of miRNA-pathway in p21 metabolism, we co-transfected hESCs with shRNAs against key players in miRNA biogenesis, proteins Argonaute2 (Ago2) and Dicer1 (Dic1). Results show that p21 protein is clearly detectable in hESCs with silenced Ago2 and Dic1 proteins while key pluripotency regulators Oct4, Nanog and Sox2 remained unaffected. Finally, to identify individual miRNAs involved in p21 regulation, we used miRNA microarrays to compare hESCs with their differentiated counterparts that show normal p21 expression (hESC-derived neural precursor cells). This approach revealed that several miRNAs upregulated after DNA damage in hECS are also expressed specifically in this cell type. In particular we identified members of the miRNA family miR-302 (miR-302s) that were also previously linked to the maintenence of stemness in hESCs. To test if miR-302s function as post-transcriptional regulators of p21 protein expression, we co-transfected undifferentiated hESCs with silenced miRNA pathway with miR-302s miRNA mimics. Importantly, matured miRNAs of the miR-302 family repeatedly rescued by about 50% the effect of global inhibition of miRNA synthesis on the level of p21 protein ($p<0.05$). We thus conclude that members of the miRNA family miR-302 are specific regulators of p21 protein in undifferentiated hESCs. Conclusion: The described mechanism explains the lack of p21 protein in hESCs before and after the DDR and elucidates the role of miRNAs in regulation of one of the most important molecular pathways governing the G1/S transition and cell cycle checkpoint in undifferentiated hESCs. Supported by IGA MZCR NT11218-6/2010, MSM0021622430 and by European Regional Development Fund (FNUSA-ICRC, No. CZ.1.05/1.1.00/02.0123)

Poster Board Number: T-3012

OVERCROWDING AND STARVATION: CULTURE DENSITY AND DNA DAMAGE IN HUMAN EMBRYONIC STEM CELLS.

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Worldwide research has uncovered genomic instability during long-term culture in human Embryonic Stem Cells (hESCs), casting doubt on the safety of these cells in cell therapy and the reproducibility of experimental results. An improved understanding of the effect of culture conditions on hESCs might contribute to the preservation of genomic integrity, and thus safeguard their scientific and clinical value. In high-density cultures, fewer nutrients are present per cell, possibly leading to a shortage of substrates for nucleotide synthesis. A lack of nucleotides could then lead to stalled replication forks, causing an increase in double-stranded DNA breaks (DSBs), and to intra- and inter-chromosomal rearrangements as a result of mis-repair. To test this hypothesis, we plated 3 different hESC lines (VUB07, VUB14 and VUB31) in 4 different densities increasing from 2 ± 0.7 colonies per cm^2 to semi-confluency. We measured a larger decrease in aspartate and glutamine (both essential for nucleotide synthesis) concentration in media of dense cultures (preliminary data), and a more than five-fold increase of lactate concentration up to 14.8 ± 2.2 mmol/L, which is described to have negative effects on the hESCs metabolism. We have also found a significant increase in the mean number of γH2AX foci per cell, the earliest marker for DSBs, and a 3-fold relative increase in the fraction of cells with more than 30 foci in the most dense condition compared to the least dense. In addition, we demonstrated a 110% increase in DNA fragmentation as shown by a single cell gel electrophoresis (COMET) assay, and preliminary data suggests an increase of more than 50% of the incidence of segmental chromosomal aberrations as shown by FISH. Our preliminary data suggest a correlation between culture density and the occurrence of DNA breaks and segmental aberrations in hESC cultures, although we still have to perform functional experiments to determine which factors are decisive for the observed effects. Nevertheless, bearing in mind that we find these substantial differences after 1 passage of 5 days, the long-term effect of culture density could have a strong impact on the genetic stability of hESC cultures.

Poster Board Number: T-3013

THE ROLE OF ABCG2 MULTIDROG TRANSPORTER DURING STRESS CONDITIONS IN HUMAN EMBRYONIC STEM CELL LINE

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The ABCG2 multidrug transporter is known to function as an important mechanism limiting cellular accumulation of various compounds. ABCG2 is widely distributed in normal tissues, moreover, it has also been indicated that ABCG2 confers the SP cell phenotype both in human and mouse progenitors and tumor cells. These findings indicate that ABCG2 expression is a conserved feature of tissue stem cells. Earlier we have shown a heterogeneous expression of ABCG2 in various pluripotent human embryonic stem cell (HESC) lines. Although the role of ABCG2 protein in evolution of multidrug resistant phenotype is well studied, the data about the substrates and function of this protein under normal physiological conditions is still unresolved. In this study we investigated ABCG2 expression in HESC cultures during different stress conditions. We examined the response of these cells to toxic effects caused by mitoxantrone or UV light exposure, measuring cell death and ABCG2 expression by flow cytometry. When the cells were exposed to these treatments causing partial cell death, after 72 hours we could not detect an induction of the overall ABCG2 expression but most of the surviving cells were those expressing ABCG2. When we studied the effects of moderate oxidative stress conditions by using low concentration of H2O2 (150 μM), cell death was negligible and after 72 hours ABCG2 expression was unchanged. However, at the early time points (3-6 hours) of H2O2 treatment the internalization of ABCG2 protein from the plasma membrane was observed by immunostaining and confocal microscopy. This internalization effect was reversible after 24 hours. In light of these results we suggest that a dynamic balance of ABCG2 expression at the population level has an advantage to promptly respond to changes in the cellular environment. Such an actively maintained heterogeneity of ABCG2 expression might be evolutionary favorable to protect valuable sanctuaries such as embryonic stem cells.

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LAMININ-521 PROVIDES AN EASY, BIOLOGICALLY RELEVANT, AND RELIABLE METHOD TO CULTURE HUMAN PLURIPOTENT STEM CELLS COST-EFFECTIVELY

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Human pluripotent stem cells (PSCs) are promising cell sources in the clinical development of regenerative medicine for disorders such as spinal cord injuries, type I diabetes, cardiovascular- and Parkinson's disease. One of the major hinders for creating stem cell-based regenerative therapy has been the many technical problems associated with the basic culturing of PSCs in a manner acceptable for regulatory authorities. Culturing PSCs such as embryonic stem cells (ESCs) has been considered difficult, labor-intense and highly variable depending on subjective measures. Current coating methods usually involve undefined feeders or mouse tumor protein extract BD Matrigel™ Matrix on which PSCs grow as heterogeneous colonies with spontaneous differentiation contaminating the cultures. There are a few defined and animal protein-free substrate alternatives such as Synthemax™ surface and CELLstart™ matrix, which together with media such as TeSR™2 medium generates a culture system devoid of animal proteins and with defined components.

A possible solution to these problems is the use of proteins in the extracellular matrix that have a major role of *in vivo* cell niches. Even though there are many different proteins in the basement membrane, the laminins are the only ones expressed tissue-specifically. Laminins are large heterotrimeric glycoproteins situated in the basement membrane in the extracellular matrix surrounding all tissues and organs in the body. They are important in cellular processes such as proliferation, migration and differentiation. There are 16 different isoforms and data indicate that different cell types prefer different laminins. Recently, it has been shown that when culturing human PSCs on human recombinant laminin-511, the cells grow as a homogenous monolayer, which makes experimental data more reproducible. In addition, the human PSCs on laminin-511 grow with many available xeno-free media such as NutriStem™ XF/FF, RegES™ and TeSR™2. Most commonly though human PSC cultures are still passaged by cutting the colonies into pieces. The plating of human ESCs as single cells after passaging requires the addition of an inhibitor of apoptosis such as ROCK. Here we show that upon cultivation of human ESCs on laminin-521 cells proliferate significantly faster compared to cultures on either Synthemax™ surface or BD Matrigel™ Matrix. They can be passaged and re-plated as single-cells using enzymatic detachment of cells with similar survival without addition of ROCK inhibitor compared to control. The human ESCs on laminin-521 even survive low single-cell plating density and grow as a homogenous monolayer without almost any spontaneous differentiation. One possible explanation to the effect laminin-521 has on cultivated human PSCs is that it is one of the first proteins expressed already by the 4-cell stage embryo and, thus, supports pluripotency in human ESCs by activating important cell signaling pathways. In conclusion, laminin-521 allows for simple enzymatic single-cell passaging, without the need for extensive training, and with a much higher expansion rate than other alternatives, saving both time and money by e.g. reduced medium costs. Laminin-521 is a biologically and regulatory superior cell culture substrate that can help make stem cell therapy a future reality.

Poster Board Number: T-3015

CHARACTERIZATION OF XCI IN A HESC LINE DERIVED UNDER PHYSIOLOGICAL LEVELS OF OXYGEN

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Murine embryonic stem cells (mESC) have been successfully used as a model for understanding molecular events in embryo development, particularly the initiation of X chromosome inactivation (XCI). Nevertheless, many different lines of female human ESC (hESC) have shown some degree of XCI even when in their undifferentiated state. Silva et al. (PNAS, 105(12): 4820-5, 2008) classified female hESC lines into three different categories: class I - lines that exhibit two active X chromosomes (resembling female mESCs) and are capable of inactivating one X chromosome upon differentiation; class II - lines that show a consistent number of XIST RNA positive cells even when in their undifferentiated state, suggestive that XCI has already been completed; and class III - lines that show some degree of XCI but had lost the ability of expressing XIST even when differentiated. The great majority of the hESC lines derived to date fall into class II and Class III categories, and many authors suggested that this could be attributed to culture conditions, especially the way these cells are handled and the level of stress to which they are submitted. However, in a recent work, Lengner et al. (Cell, 141(5): 872-83, 2010) showed that the use of physiological levels of oxygen (5% O2) during the derivation and maintenance of hESC is critical in preserving the pre-XCI state of hESCs. Here we describe the establishment of a new line of hESC in 5% O2, and the characterization of its state of XCI. This line, named BR5, was derived from a surplus embryo generated for reproduction purposes in an IVF clinic, and was donated for research under informed consent. The whole blastocyst was plated on a MEF cell layer, and since then was kept under hypoxia conditions. After few passages, the cells were transferred to matrigel and mTeSR1. The undifferentiated cells expressed pluripotency markers, and were able to differentiate into cell types that are representative of the three embryonic germ layers both in vitro and *in vivo*. The status of XCI in these cells was verified by XIST RNA-FISH, and by real time RT-PCR for XIST and some X-linked genes that are submitted to inactivation. Our results demonstrate that approximately 20% of both undifferentiated and differentiated cells express XIST, as evidenced by RNA-FISH and real-time RT-PCR. Additionally, accumulation of H3K27me3 (histone H3 lysine 27 trimethylation, a hallmark of XCI) was observed in nearly 100% of both undifferentiated and differentiated cells. Therefore, the BR-5 cells are apparently in the transition of class II to class III. Our data indicates that the establishment and maintenance of hESCs in 5% O2 is not sufficient to keep the cells in a pre-XCI state.

Poster Board Number: T-3016

HESC EXPANSION AND STEMNESS ARE INDEPENDENT OF INTERCELLULAR COMMUNICATION THROUGH CONNEXIN 43 BETWEEN HESC AND FEEDER CELLS

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Human embryonic stem cells (hESC) have attracted a great interest in the fields of regenerative medicine, tissue engineering,

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cell-based therapy because of their self-renewal and pluripotent properties. For hESC expansion, many researchers have followed conventional technique using feeder cells despite feeder free culture technique. Classical studies demonstrated that gap junctional intercellular communication (GJIC) is thought to play an important role in hESC survival and differentiation. Indeed, it has been reported that Connexin 43 (Cx43, one of the major gap junction protein) are crucial in maintaining the pluripotency of hESCs. However, the role of GJIC between hESC and feeder cells is unclear and has not been reported. In this study, we examined whether the direct interaction through Cx43 between hESCs and feeder cells influences on the maintenance of hESC stemness. Until 20 passages, the hESCs cultured on Cx43 down-regulated feeder cells sustained normal morphology, proliferation and stemness showing pluripotent markers (AP activity, Oct4, Sox2, Nanog expression). These results demonstrate the direct interaction through Cx43 between hESCs and feeder cells would not be an important factor to maintain cell growth and stemness of hESC. Keywords: hESC expansion, Feeder cells, Gap junction, Connexin 43, hESC stemness

Poster Board Number: T-3017

SMALL MOLECULE-ASSISTED; LINE-INDEPENDENT MAINTENANCE OF HUMAN PLURIPOTENT STEM CELLS IN DEFINED CONDITIONS

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Human pluripotent stem cells (hPSCs) are conventionally grown in a mouse feeder cell-dependent manner. Chemically defined culture conditions are, however, desirable not only for potential medically oriented applications but also for investigating mechanisms of self-renewal and differentiation. In light of the rather high complexity and cost of existing defined hPSC culture systems, we have systematically evaluated over 20 potential media ingredients. Only components that reproducibly gave beneficial effects were ultimately combined to yield a simple and cost-effective formulation termed FTDA. This xeno-free medium is based on mimicking self-renewal factor activities present in mouse embryonic fibroblast-conditioned medium, with all factors applied at minimal dosages. Additionally, selected small molecules served to specifically suppress typical types of spontaneous differentiation seen in hPSC cultures. FTDA medium was suitable for the generation of human induced pluripotent stem cells and enabled robust long-term maintenance of diverse hPSC lines including hard-to-grow ones. Comparisons with existing defined media suggested reduced spontaneous differentiation rates in FTDA. Our results hence imply that using supportive factors at minimal concentrations may still promote robust self-renewal and preserve pluripotency of hPSCs. Moreover, by modifying the growth factor composition but maintaining its basal ingredients, directed differentiation along all three germ layers could selectively be initiated. Thus, FTDA not only presents a cost-effective alternative for robust large-scale expansion of hPSCs but also a versatile media platform for studying mechanisms of self-renewal and differentiation.

Poster Board Number: T-3018

RE-ESTABLISHMENT OF GAP JUNCTIONAL INTERCELLULAR COMMUNICATIONS DURING REPROGRAMMING TO PLURIPOTENCY AND DIFFERENTIATION

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Gap junctional intercellular communication (GJIC) has been described in embryonic stem cells (ESCs) and various somatic cells. GJIC has been implicated in the regulation of cell proliferation, self-renewal, and differentiation. Recently, a new type of pluripotent stem cells was generated by direct reprogramming of somatic cells. Here, for the first time, we show that during reprogramming events GJIC is dramatically reduced and re-established again upon reaching complete reprogramming. The opposite process of cell differentiation from the pluripotent state leads to the disruption of GJIC between pluripotent and differentiated cell subsets. However, GJIC is subsequently re-established *de novo* within each differentiated cell type, forming "communication compartments" within a histotype. Our results provide the important evidence that the presence of functional gap junctions is an additional physiological characteristic of somatic cell reprogramming to the pluripotent state and differentiation to the specific cell type.

Poster Board Number: T-3019

TRANSCRIPTIONAL REGULATION MEDIATED BY THE DNA REPAIR COMPLEX XPC-RAD23B-CETN2 IN MURINE EMBRYONIC STEM CELLS

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Embryonic stem cell (ESC) self-renewal and pluripotency are transcriptionally controlled by the "core" transcription factors Oct4, Sox2 and Nanog together with cofactors, chromatin regulators, non-coding RNAs and other terminal effectors of developmental signalling pathways. Identifying components of these complex circuitries and understanding their interplay provides the knowledge base to promote the efficacious deployment of ESCs and improves methods for induction and differentiation of pluripotent stem cells. We recently identified a new stem cell transcriptional coactivator (SCC) required for synergistic activation of the Nanog promoter by Oct4 and Sox2. Biochemical characterization of SCC revealed it to be the nucleotide excision repair complex Xpc-Rad23b-Cetn2, although the DNA-repair activity is not required for its transcription function. SCC interacts directly with Oct4 and Sox2 on their target genes, and its depletion compromises both ESC pluripotency and somatic cell reprogramming. To further dissect the transcriptional network orchestrated by the SCC complex in ESCs, we used chromatin immunoprecipitation followed by high-throughput sequencing to map regions bound by Rad23b in the mouse genome. Rad23b peaks are enriched around the transcription start site (TSS) of active and inactive genes, suggesting a role for SCC in both activation and repression of transcription. Around 60% of the high-confidence Rad23b binding sites co-localize with Oct4/Sox2-occupied regions, and these are predominantly at distal enhancer elements of active genes. Sequence analysis of Rad23b peaks confirmed that Oct4/

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Sox2 recognition motifs are the most significantly enriched at these sites. Interestingly, the majority of genes targeted by Rad23b at core promoter proximal sites (±500bp around the TSS) are also marked by distal peaks, suggesting that SCC might participate in the crosstalk between distal enhancers and the basal transcription machinery. Intriguingly, a significant proportion of Rad23b binding sites are also co-occupied by Mediator and cohesin, two components thought to mediate gene activation by DNA-looping. A gene ontology classification of Rad23b targets revealed a weak over-representation of transcription regulators among the active genes, but a strong bias towards Polycomb-repressed developmental genes, regardless of Oct4/Sox2 association, hinting at Oct4/Sox2-independent functions for SCC/Rad23b. Indeed, overlap of Rad23b binding sites with other key ESC transcription factors (Stat3, c-Myc, n-Myc, Klf4, Zfx, E2f1, Tcfcp2l1, Essrb) showed variable degrees of co-occupancy. Thus our initial genome-wide analysis may have uncovered previously unrecognized and potentially widespread roles for SCC in coordinating ESC-specific transcriptional programs, both at repressed and active gene loci, while also laying the basis for a potential novel connection between DNA-repair and pluripotency.

Poster Board Number: T-3020

DEFINING THE ROLE OF REST IN REGULATING HUMAN EMBRYONIC STEM CELL FATE

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Human embryonic stem cells (hESCs) have the unique ability to undergo self-renewal and are pluripotent, which enables them to differentiate into all three germ layers. Harnessing these properties could result in significant therapeutic outcomes for repair or cell replacement in many disease settings. However, many hurdles remain prior to use of these cells in cell-based therapies including lack of understanding of what signals regulate pluripotency and how to maintain stability of hESCs. OCT4, NANOG and SOX2 are transcription factors that regulate multi-gene networks involved in control of cell fate decisions important for maintaining self-renewal and stability of hESCs. RE1-Silencing Transcription Factor (REST) is one of the direct targets of this network which is a well known transcriptional repressor. REST acts by binding to RE1 sites in greater than 1900 genes in the genome. The main role of REST is to act as a transcriptional repressor in non-neuronal cell types to repress neuronal differentiation. This repression has been shown in some but not all mouse embryonic stem cell studies, however, its role in hESCs is not clear. To examine the role of REST in hESCs, we have performed lentiviral mediated knockdown of REST (REST KD) in three different cell lines and we are currently evaluating the effects of loss of REST on hESC self-renewal, differentiation, genetic stability and survival. REST KD hESCs seem to exhibit enhanced self-renewal and survival capacity compared to controls. We have also found that three out of four hESC lines with REST KD showed genetic instability, whereas control hESC lines were karyotypically normal. To determine the gene networks involved in this instability, we are currently examining candidate genes differentially expressed upon REST KD by performing qPCR of individual REST targets. We are also evaluating the expression of lineage markers in embryoid bodies from REST KD and control cells in order to examine the effects of REST on differentiation. Upon REST KD hESCs exhibit a biased towards endodermal lineage. To examine the role of REST in maintaining genomic stability, we are analyzing H2AX accumulation after DNA damage of hESCs. In summary, our work has shown that REST is a regulator of hESC genetic stability and elucidating the targets mis-regulated upon REST KD could shed light on the gene networks regulating instability in pluripotent stem cell cultures and improve our ability to stably expand and differentiate hESCs for use in regenerative medicine.

Poster Board Number: T-3021

INHIBITION OF P53 SUPPRESSES SELF-RENEWAL OF MURINE EMBRYONIC STEM CELLS

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Embryonic stem (ES) cells have the remarkable capacity to divide indefinitely while retaining their wide range differentiation potential, and they represent a promising source for cell transplantation therapies. Recent studies have reported the roles of p53 in suppressing the self-renewal of ES cells after DNA damage and block the reprogramming of somatic cells into induced pluripotent stem cells (iPSCs). However, to date no evidence has been presented to support the function of p53 in unstressed ES cells. In this study, we investigated the effect of pifithrin (PFT)-α, an inhibitor of p53 transactivation, on self-renewal of ES cells. Our results revealed that treatment of ES cells with PFT-α resulted in the inhibition of ES cell propagation in a dose-dependent manner, as indicated by a marked reduction in the cell number and colony size. Furthermore, PFT-α caused a cell cycle arrest and a significant reduction in DNA synthesis. In addition, inhibition of p53 reduced the expression levels of cyclin D1 and Nanog. These findings indicate that p53 pathway in ES cells rather than acting as an inactive gene, is required for ES cell proliferation and self-renewal.

Poster Board Number: T-3022

STEM-CELL SPECIFIC REGULATION OF RRNA PROCESSING IN MOUSE AND HUMAN EMBRYONIC STEM CELLS

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Embryonic stem cells (ESCs) are characterized by their unusual cell cycle and in particular abbreviated G1 phase. As ribosomal biogenesis is typically tied to checkpoints regulating G1 transition, we investigated how ribosomal biogenesis is regulated in pluripotent and differentiated mouse and human ESC. We find that as expected, transcription of ribosomal DNA is higher in ESC compared to differentiated ESC. However, processing of primary 47S rRNA precursor to cytoplasmic 28S RNA was significantly less efficient under stem cell conditions in both mouse and human ESC. Relative to their differentiated counterparts, we find that rRNA processing appears to be delayed at an intermediate step between 32S to 28S. Mechanisms contributing to delayed processing were investigated including nuclear export, limiting rRNA processing proteins, and others. We present further evidence that developmental regulation of rRNA processing is not common event in most tissues during development and propose that this process represents a novel, evolutionarily conserved, stem-cell specific event contributing to the pluripotency and self-renewal of ESC.

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Poster Board Number: T-3023

INDUCED PLURIPOTENT STEM CELLS DERIVED FROM ADULT MOUSE TESTIS DIFFERENTIATED INTO MESODERM, ENDODERM AND ECTODERM

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Induced pluripotent stem cells are cells that were originally from adult tissues, but have been enforced to produce proteins that are thought to be essential for the pluripotency of human embryonic stem cells. By making cells express these embryonic stem cell proteins, adult cells can be created that look and act nearly identical to hESCs. The ability to reprogram a somatic cell and restore its pluripotency has been one of the most significant fields that created new opportunities for treating many of the human diseases. Most of the research that has been done on reprogramming somatic cells was done with the use of viral vectors that deliver certain transcription factors (Oct4, Sox2, c-myc, Klf4, and nanog) which initiate the pluripotency of a somatic cell. Using viral vectors has showed some disadvantages such as this viral genome may integrate the reprogramming factors into the host genomes and may increase the risk of tumor formation. Throughout our research we have used spermatogonial stem cells which are extracted from mice's testicles to convert them into induced pluripotent stem cells. Spermatogonial stem cells had been reprogrammed without the use of any viral vectors; these cells were reprogrammed by the addition of the appropriate growth factor and embryonic stem cells medium. The resulted induced pluripotent stem cells were then differentiated to the three germ layers and organ lineage which are the endoderm (islet like cells), mesoderm (cardiac cells) and ectoderm (epidermal cells). Our results have shown totally reprogrammed embryonic stem cells which were then differentiated into islet like cells, cardiac cells and epidermal cells.

Poster Withdrawn

Poster Board Number: T-3025

ROLE OF KLF5 IN THE MAINTENANCE OF SUBPOPULATIONS OF MOUSE ES CELLS

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ES (Embryonic Stem) cells are derived from the blastocyst and have the potential to give rise to derivatives of each germ layer. Induced pluripotent stem (iPS) cells can be derived from lineage-restricted cells, such as fibroblasts, by forced expression of specific transcription factors. Although recent studies indicate that Krüppel-like factors (Klfs) are essential for both maintenance of ES cell self-renewal and reprogramming of somatic cells into a pluripotent state, the molecular mechanism of these processes remains unknown. Thus, understanding the molecular mechanism of ES cell self-renewal by Klfs would be important for the efficient generation of patient-specific pluripotent stem cells and for the development of regenerative medicine. Although we have showed that Klf5 is important for the self-renewal of mouse ESCs and blastocyst development, the molecular mechanism underlying these functions remains unknown. Recent studies indicate that mouse ES cells are heterogeneous and consist of Rex1 positive and negative subpopulations even in the presence of LIF. To understand the mechanism of self-renewal of mouse ES cells controlled by Klf5, we addressed the role of Klf5 in the maintenance of Rex1 positive and negative cells in mouse ES cells. By using Rex1-GFP knock-in ES cells (OCRG9), we have generated Klf5 KO and Klf5-overexpressing ES cells. We also generated ES cells which overexpress other Klf member such as Klf2, Klf4 and Klf10 to investigate the overlapped and non-overlapped functions between Klf family members. These studies will shed light on the molecular mechanism of mouse ESC self renewal controlled by Klf family members.

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Poster Board Number: T-3026

SCREENING FOR CRITICAL EPIGENETIC FACTORS INVOLVED WITH THE REGULATION OF PLURIPOTENCY AND DIFFERENTIATION USING A HOMOZYGOUS MUTANT MOUSE EMBRYONIC STEM CELL BANK

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Pluripotent cells have a unique and characteristic epigenetic signature that reflects their broad developmental potential. This epigenetic state dynamically changes during differentiation. Dramatic changes of epigenetic state also occur during reprogramming of somatic cells into induced pluripotent stem cells (iPSCs). Despite increasing evidence that epigenetic regulation is key to the maintenance of the stem cell state, detailed mechanisms associated with this are still unknown. Therefore, elucidation of the functions of epigenetic regulators (such as chromatin modifiers, transcription factors and non-coding RNAs) will help better understand the pluripotent states of embryonic stem cells (ESCs) as well as the induction of iPSCs from somatic cells. We recently reported a method to rapidly generate homozygous mutant mouse ESCs from heterozygous mutant ESCs using the conditional knockout of the Bloom's syndrome gene (Blm). This method takes advantage of the high rate of loss of heterozygosity associated with Blm-deficient condition. We have so far generated ~200 homozygous mutant ESC lines from heterozygous ESC lines. Utilizing these homozygous mutants, we are currently screening for novel epigenetic factors involved in the regulation of pluripotency and differentiation. We are also screening for unknown functions of known epigenetic factors (such as polycomb complex, DNA and histone methyltransferases, chromatin-remodelers, MBD proteins and miRNA processing factors) in the differentiation and maintenance of pluripotency.
In order to identify such factors, we compared the epigenetic state between wild type ESCs and each homozygous mutants by the following methods: (1) digest the genome of each mutant by methylation-sensitive restriction enzymes, (2) carry out immunostaining of various histone modifications, (3) quantify the expression level of various retroelements (such as IAPs, MLV, ETn-MusD, ERVK, LINEs and SINEs), (4) examine the response against inhibitors of known epigenetic modifiers. We also conducted phenotypic analysis upon differentiation induction to screen for mutant ESCs that are prone or resistant to differentiation. We could so far identify some candidate genes that might play a critical role in epigenetic regulation of pluripotency and differentiation. We plan to examine expression levels of cell lineage markers in embryoid body formation to gain insight into the role of the candidate genes in cell lineage commitment. We also plan to conduct molecular and genome-wide analysis of these candidates in order to elucidate the mechanisms of these epigenetic regulations. Our approach allows the phenotype comparison amongst many homozygous mutants under the same experimental conditions, and to identify critical epigenetic regulators involved in the regulation of pluripotency and differentiation.

Poster Board Number: T-3027

ANALYSIS AND CHARACTERIZATION OF NANOG-INTERACTING PROTEINS IN MOUSE EMBRYONIC STEM CELLS

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Embryonic Stem (ES) cells are maintained in an undifferentiated state by a gene regulatory network centred on the triumvirate of transcription factors Nanog, Oct4 and Sox2. Genome-wide chromatin immunoprecipitation studies indicate that in many cases target genes are bound by closely localised binding sites for each of these proteins, as well as additional members of the extended pluripotency transcription factor network. However, the biochemical basis of the interactions between these proteins is largely unknown, as are the mechanisms by which these interactions control ES cell identity. We recently presented an optimised method for identification of protein-protein interactions and used this to characterise an extended Oct4-centred ES cell interactome (1). Interestingly, Nanog is not present in the Oct4 interactome but it is a member of the extended protein network generated by combining the interaction data of Oct4, Esrrb, Sall4, Dax1 and Tcfcp2l1. We have used a similar approach to identify an extended Nanog interactome that includes more than 60 putative partners. Validation of interactions was obtained by co-immunoprecipitation of Nanog with putative partners. In specific cases, we show that transcription factor interactions occur independent of DNA binding. Moreover, mutational analyses have been used to pinpoint the sites of interaction on Nanog. Mutation of tryptophan residues within the Nanog tryptophan repeat (WR) abolishes some interactions. In addition, the WR (but not the W0 derivative in which all tryptophan residues are mutated to alanine residues) is sufficient to mediate Nanog binding to some partners. These data show that the tryptophans present in the WR are necessary and sufficient to mediate interaction between Nanog and such interacting partners. Likewise, amino acid residues on partner proteins required for the interaction with Nanog have been identified. Our data shed light on the biochemical nature of the interaction between Nanog and its partner proteins that are crucial in maintaining optimal mouse ES cell self-renewal efficiency.
(1) Van den Berg, D., Snoek, T., Mullin, N.P., Yates, A., Bezstarosti, K., Demmers, J. Chambers, I. and Poot, R.A. (2010) An Oct4-centred protein interaction network in embryonic stem cells. Cell Stem Cell, 6, 369-381.

Poster Board Number: T-3028

INHIBITION OF TGF-B SIGNALING COULD SUBSTITUTE THE INHIBITION OF MULTIFUNCTIONAL GSK3 IN NAIVE MOUSE EMBRYONIC STEM CELLS

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The first mouse embryonic stem (ES) cell lines generated on undefined culture conditions including serum and mouse embryonic fibroblasts (MEF) as feeder layers from blastocysts of strain 129. The feeder cells and serum were later substituted by LIF and BMP4, respectively and in this manner feeder- and serum-free culture conditions for long-term cultivation of mouse ES cells were established. However this defined media is not enough to derive ES cells from

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mouse strains other than strain 129. Although the precise reason for this problem has yet to be identified, but with usage of diapauses embryos or some small molecule (SM) inhibitors the derivation of authentic mouse ES cells from recalcitrant strains have been practicable. Especially the application of SM inhibitors of ERK1/2 (PD0325901) and GSK3 (CHIR99021), known as 2i, has provided the possibility of derivation and propagation of ES cells in a chemically defined medium from different mouse strains. However, an impressive disadvantageous of this condition is the usage of CHIR; as it is shown that this chemical or other GSK3 inhibitors induce chromosomal instability. We have recently shown that the inhibition of TGF-β signaling pathway by SM inhibitors of TGF-β type I activin receptor-like kinase (ALK) 4, 5, 7 inhibitors (such as SB431542 or SB) in combination with PD0325901 (PD), will result in highly efficient and reproducible derivation of mouse ES cells from NMRI, BALB/c, C57BL/6, DBA/2 and FVB/N that previously considered as refractory or non-permissive strains under conventional condition of mouse ES cell cultures. This 100% efficiency of derivation encouraged us to evaluate the derivation and maintenance of mouse ES cells under chemically defined media supplemented with PD+SB as an alternative way to gain pluripotency instead of the suppression of multifunctional GSK3 molecule. Our results showed that PD+SB could support the generation and long term cultivation of mouse ES cells from different mouse strains. Derived lines showed the typical morphology of ES cells and expression of pluripotency markers. Also they showed the ability of differentiation by EB formation (spontaneously differentiation), direct differentiation or chimera formation into derivatives of three embryonic germ layers. Interestingly the results of qRT-PCR showed that PD+SB not only support the pluripotency but also give rise to better expression of some pluripotency markers such as stella and extremely low or complete lack of expression of some early lineage differentiation genes such as Brachyury, Lefty1 and Lefty2 in comparison to 2i. Especially, PD+SB maintain the entirety of chromosomal stability contrary to 2i. Consequently, it seems that PD+SB could be introduced as suitable substitute for 2i in derivation and cultivation of rodent ES cells in chemically defined conditions.

Poster Board Number: T-3029

INVOLVEMENT OF ETS-RELATED TRANSCRIPTION FACTORS ETV4 AND ETV5 IN PLURIPOTENCY AND PROLIFERATION OF MOUSE EMBRYONIC STEM CELLS

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Pluripotent embryonic stem (ES) cells maintain an undifferentiated state in the presence of LIF. Several transcription factors including Oct3/4 are involved in the self-renewal of ES cells. Recent high-throughput, genome-wide analyses revealed that Ets-related transcription factors ETV4 and ETV5 are downstream molecules of Oct3/4 in ES cells. In the present study, we investigated roles and functions of ETV4 and ETV5 in ES cells. ETV4 and ETV5 are specifically expressed in undifferentiated ES cells, and suppression of Oct3/4 resulted in down-regulation of ETV4 and ETV5. Reporter and electrophoresis mobility shift assays identified a binding site for Oct3/4 in the enhancer region of the ETV5 gene, suggesting that ETV5 is one of the direct downstream target genes of Oct3/4 in ES cells. ETV4 and ETV5 have similar molecular structures and exhibit overlapping expression, indicating that ETV4 and ETV5 have redundant activities. Therefore, next we compared and examined phenotypes between wild-type (WT) and ETV4/5 double knockout (dKO) ES cells. WT ES cells form compact colony, which is the typical feature of undifferentiated ES cells; whereas, cell shapes of dKO ES cells were flat like epithelial cells. Of note, expression levels of self-renewal marker genes (Oct3/4, Nanog and Dax1) were similar between these two cell lines, suggesting that ETV4 and ETV5 are dispensable for the self-renewal of ES cells. We also examined differentiation ability of dKO ES cells with the system of embryoid bodies (EBs) in suspension culture which mimics early embryogenesis *in vitro*. dKO ES cells did not form EBs but grew as adherent cells. Interestingly, these cells showed enhanced expression of extra-embryonic endoderm (ExEn) marker genes including Gata4, Gata6, Sox17 and HNF3β and reduced expression of other linage marker genes (T and Fgf5), suggesting that dKO ES cells are prone to differentiate into ExEn and defective in pluripotency. Since ETV4 and ETV5 are involved in proliferation of cancer cells, we compared growth of these ES cells. Direct cell counting and WST-1 assay demonstrated that proliferation of dKO ES cells was significantly decreased when compared to that of WT ES cells, and cyclin D1 expression level was downregulated in dKO ES cells. Taken together, these results indicate that ETV4 and ETV5 are involved in pluripotency and proliferation of mouse ES cells.

Poster Board Number: T-3030

E-CADHERIN AND, IN ITS ABSENCE, N-CADHERIN PROMOTES NANOG EXPRESSION IN MOUSE EMBRYONIC STEM CELLS VIA STAT3 PHOSPHORYLATION

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We have recently shown that loss of E-cadherin in mouse embryonic stem (mES) cells results in significant alterations to both the transcriptome and hierarchy of pluripotency-associated signalling pathways. Here we show that E-cadherin promotes Klf4 and Nanog transcript expression in mES cells via STAT3 phosphorylation and that β-catenin, and its binding region in E-cadherin, is required for this function. To further investigate the role of E-cadherin in LIF-dependent pluripotency, E-cadherin null ($Ecad^{-/-}$) mES cells were cultured in LIF/BMP supplemented medium. Under these conditions, $Ecad^{-/-}$ mES cells exhibited restoration of cell-cell contact and STAT3 phosphorylation and upregulated Klf4, Nanog and N-cadherin. Abrogation of N-cadherin using an inhibitory peptide caused loss of pSTAT3, Klf4 and Nanog in these cells, demonstrating that N-cadherin supports LIF-dependent pluripotency in this context. We therefore identify a novel molecular mechanism linking E- and N-cadherin to the core circuitry of pluripotency. This mechanism may explain the recently documented role of E-cadherin in efficient induced pluripotent stem cell reprogramming.

Poster Board Number: T-3031

EPIGENETIC REGULATION OF GERMLINE GENES IN PLURIPOTENT CELLS

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While there has been extensive characterization of the transcriptional program underlying pluripotency in embryonic stem (ES) cells, much less is known about the pluripotency transcription network *in vivo*. To address this, we transcriptionally profiled two pluripotency-associated cell populations present in the mouse embryo: the inner cell mass (ICM) of the blastocyst, the cells from

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which ES cells are derived, and primordial germ cells (PGCs), the embryonic germline. This analysis reveals strikingly similar transcriptional profiles between PGCs, ICM, and ES cells. We noticed that many germline genes were also expressed in ES cells, albeit at lower levels, and validated the data by qRT-PCR and IF. Two important questions arise from these observations: 1) How is the expression of germline genes regulated in ES cells? We hypothesize that these genes are regulated epigenetically. We developed culture conditions in which the expression of germline genes is induced in ES cells, while maintaining the undifferentiated state. We are investigating the various possible epigenetic mechanisms that may regulate induction of germline genes in ES cells. Intriguingly, DNA de-methylation correlates well with germline gene induction. We are carrying out further studies on the mechanisms that regulate the expression of germline genes in ES cells. 2) What is the functional significance of the expression of germline genes in ES cells? It has been proposed that during ES cell derivation, under some culture conditions, ES cells may pass through a germ cell state. Our data suggest that during ES cell propagation a state permissive for germline gene induction is maintained, and can be modulated by culture conditions. In particular, we find that the "2i" medium, which maintains mouse ES cells in a naïve ground state, can respond robustly to conditions that induce germline genes. However epiblast stem cells (EpiSCs) and embryonic fibroblasts (MEFs) appear to not be capable of inducing germline genes in response to the same culture manipulations. We are studying this differential response further. In addition, we are investigating the effect that manipulations that lead to germline gene induction have on mouse iPS cell generation. We have found that these manipulations highly increase the efficiency of iPS cell generation, though it remains to be determined if germline genes have a functional role in the process. Our most recent results will be presented.

Poster Board Number: T-3032

A RECESSIVE GENETIC SCREEN FOR PLURIPOTENCY REGULATORS USING THE HOMOZYGOUS MUTANT MOUSE EMBRYONIC STEM CELL BANK AND ITS APPLICATION TO REPROGRAMMING STUDY

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Recessive genetic screening is a powerful method to identify novel genetic pathways. However, it requires large-scale generation of homozygous mutant cells, which is difficult in mammalian cells. We recently reported a method to rapidly generate homozygous mutant mouse embryonic stem cells (ESCs) from heterozygous mutant ESCs using the Bloom's syndrome gene (Blm) conditional knockout model. This method takes advantage of the high rate of loss of heterozygosity due to Blm-deficiency. Combined with gene trap technology, we have so far generated ~2,000 heterozygous mutant ESC lines. For the induction of homozygous ESC lines, we preferentially selected genes for which corresponding homozygotes were poorly characterized or embryonically lethal. We also placed priority on genes that seem to be involved in pluripotency regulation, e.g., high expression in early embryos, lincRNAs, or interaction with core pluripotency factors such as Oct3/4 and Nanog. To date, we have generated ~200 homozygous mutant ESC lines from heterozygous ESC lines. Here we present our approach for screening pluripotency regulators using our homozygous ESC bank and its application to reprogramming. We screened for homozygous ESC lines that are either prone or resistant to differentiation, or defective in self-renewal. We have identified several mutants that have been reported to show such phenotype, e.g., components in polycomb complex, upstream regulators of Erk pathway, and an essential factor in microRNA biogenesis. We have also identified mutant ESC lines that have not been reported previously, such as an interactor of Nanog showing substantial defect in self-renewal. Factors regulating ESC pluripotency may be involved in reprogramming process. We are currently analyzing the role of N-myritoyltransferase (Nmt) in reprogramming. There are two members of Nmt in mammalian cells, Nmt1 and Nmt2. Expression of Nmt1 is higher in ESCs and Nmt1-homozygous mutant ESCs were resistant to differentiation. As an experimental model to study reprogramming process, we induced mouse epiblast stem cells (EpiSCs) to naïve state. Induction to naïve state was inefficient by treatment with 2i (Mek and Gsk3 inhibitors). Inhibition of Nmt activity in the presence of 2i generated ESC-like dome-shaped compact colonies with high expression of Rex1 and Dppa3, and with nearly undetectable level of Fgf5. Naïve state was confirmed by the generation of chimeric mice. These results support the principle of our approach and we are continuing to identify novel factors regulating ESC pluripotency and reprogramming to naïve state.

Poster Board Number: T-3033

REGULATION OF STEM CELL PLURIPOTENCY BY BET PROTEINS

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The pluripotent state of embryonic stem cells (ESC) is maintained by high-level of expression of stem cell-specific transcription factors including Oct-3/4, Sox2, Klf4 and c-Myc (OSKM). ESC possess an unusual "open" chromatin conformation that allows readily access to the genome by the transcription machinery thus resulting in elevated plasticity. Transcription factors and chromatin remodeling complexes are key determinants of ESCs identity. The Bromodomain (BRD)-and extraterminal domain (BET) family of proteins regulates chromatin dynamics through modulating acetylation-mediated protein-protein and protein-chromatin interactions in control of gene transcription. We sought to investigate the role of BETs in governing ESCs self-renewal and pluripotency. Both, small-molecule chemical inhibition and downregulation of BET by RNA interference effectively reduced the expression of OSKM genes in human and mouse ESC and the number of undifferentiated-alkaline phosphatase-positive colonies. Furthermore, chromatin immunoprecipitation (ChIP) experiments of compound-treated ESC revealed displacement of BrD-containing proteins from stem cell genes promoters, with concomitant reduction of active histone marks and RNA polymerase recruitment, thus revealing a role for BET proteins in the stem cell genes transcriptional network. Changes in cell morphology and cell cycle arrest accompanied BRD inhibition. RNA sequencing analysis of compound-treated ESCs revealed differentially expressed genes of the PDGF, TGFβ and Wnt signaling pathways. Epithelial to mesenchymal transition (EMT) markers and neuroectodermal lineage commitment increased following compound treatment, without any overt effect on cell viability or apoptosis. In addition, using an ESC-based reprogramming system, we found that BET inhibition impairs somatic reprogramming of MEFs to iPS. All together, these findings unravel a key role for BET proteins in the maintenance of the embryonic stem cell state.

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Poster Board Number: T-3034

COMPREHENSIVE ANALYSIS OF GENOMIC IMPRINTING IN PARTHENOGENETIC AND ANDROGENETIC MOUSE EMBRYONIC STEM CELLS

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To unbiasly survey the mouse genome for imprinting genes in the early embryo, we profiled RNA transcriptome in both parthenogenetic and androgenetic mouse embryonic stem cell (mESC) lines to identify parental-specific transcripts. We uncovered a novel set of protein-coding and non-coding RNAs that show parent-of-origin expression, indicating that these genes are imprinted in mESCs. Significantly, we identified many paternally expressed genes involved in differentiation and development, including an entire Hox cluster. Furthermore, small RNA-Seq demonstrated some miRNAs are also regulated by genomic imprinting, such as miR-675 which is imbedded within the maternally expressed H19 gene. Because imprinting control regions contain differential methylation regions that are regulated by DNA methylation, we further tested whether these imprinted genes are deregulated in demethylated mESCs deficient of all known DNA methyltransferases. We found that mESCs null of DNA methylation show deregulation in a large subset of predicted imprinting genes. Interestingly, for the list of known imprinting genes, many of them are either not expressed or do not show mono-allelic expression in mESCs, suggesting that mESCs have a unique repertoire of expressed imprinting genes. Together, our study provides a comprehensive analysis of imprinted genes in mouse embryonic stem cells and validates the essential role for DNA methylation in genomic imprinting.

Poster Board Number: T-3035

THE POWER OF CELL AUTONOMOUS GROWTH IN THE ABSENCE OF ERK-SIGNALLING IS ACQUIRED JUST BEFORE IMPLANTATION

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One of the defining features of naïve pluripotent mouse embryonic stem cells (ESC) is their ability to thrive in the absence of Erk signaling. Dual inhibition of Gsk3 and Mekk (2i) with the optional use of leukaemia inhibitory factor (LIF) has been proposed to "capture" cells in a naïve ground state directly from late preimplantation epiblast. Nevertheless, the process of ESC derivation remains a black box. Even in defined conditions, intact inner cell masses (ICM) provide themselves with unknown paracrine signals as well as intercellular interactions. Also, this micro-niche might "carry" cells, unable to respond to 2i plus LIF, to a later developmental stage, when they are able to do so. To eliminate all of these disturbances, we analysed ESC derivation at a single cell level in Gelatin coated, individual 96wells using defined media. We set out to identify at which stage ICM/epiblast cells become responsive to 2i plus LIF during early embryonic development. The ability of dissociated ICM/epiblast cells to give rise to primary ESC colonies was assayed from an early, cavitating blastocyst (E3.25) to late, periimplantation blastocyst stages (E4.5). After analysis of more than 1500 cells, we found that only single cells isolated from E3.75-E4.5 embryos were able to give rise to colonies in these minimal conditions. A subset of these primary colonies was expanded and injected into blastocysts. All of the ESC lines analysed contributed to chimeras, including the germline. In contrast, no colonies could be derived from individual cells at a slightly earlier (E3.25-E3.5) developmental stage. Earlier ICM cells had a tendency to remain in culture for prolonged periods of time without undergoing apoptosis or cell division. Notably, primary outgrowths were efficiently obtained from whole ICMs of earlier stages (E3.25-E3.5). We speculate that these outgrowths are the result of paracrine signalling as well as extensive cell-cell interactions. As ESC can be maintained in ground-state conditions by culture in 2i alone, we interrogated the role of LIF in the process of single ICM cell derivation. Without LIF, only a small fraction of single cells was able to give rise to primary ESC colonies in 2i conditions. Also, it further narrowed the embryonic stage from which colonies could be derived down to E4.25-4.5. These observations are consistent with previous reports that LIF is an important factor for ESC clonogenicity. A possible explanation could be that LIF reinforces molecular circuitries essential for naïve pluripotency, including its direct target Klf4. Further experiments will be carried out to clarify the role of LIF in this context. In conclusion, our results suggest that the founder cells of the *embryo proper* become responsive to 2i plus LIF only about 18h before blastocyst implantation. This supports the hypothesis that naïve pluripotency is a transient state, which can be directly captured *in vitro*.

Poster Board Number: T-3036

ESTABLISHMENT OF GERMLINE-COMPETEN ES CELLS FROM NZB/BINJ MICE

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As mouse embryonic stem (ES) cells can differentiate into almost any type of cell in the body, including germline lineage, ES cells have been routinely used to generate gene-targeted mice. A wide range of mouse strains have been characterized in detail and widely used in biomedical research according to phenotypic requirements. Nevertheless, germline-competent ES cells have only been established from a few strains. Therefore, we aim to establish ES cells derived from various mouse strains. Here, we attempted to establish germline-competent ES cells from NZB mice with 4 different culture media. First, we performed a comparative investigation of effects of leukemia inhibitory factor (LIF) at high concentration. In previously study, we reported that although addition of 1,000 IU/ml LIF was effective in derivation of C57BL/6 ES cells, 5,000 IU/ml LIF was supported the effective establishment of germline-competent ES cells from BALB/c mice. However, in NZB mice, increasing the concentration of LIF in medium was not effective because established ES cells showed somatic pluripotency but are incapable of contributing to the germ lineage. Second, we investigated the effects of 2 signal inhibitors (2i). 2i is a composed of a glycogen synthase kinase 3 inhibitor (CHIR99021) and a mitogen-activated protein kinase kinase inhibitor (PD0325901), and it is already reported that 2i is effective in establishment of germline-competent ES cells from refractory strains (i.e. NOD mice) and species (i.e. rats). Unexpectedly, when we used ES cell culture medium containing 1,000 IU/ml LIF and 2i, established NZB ES cells contributed to the somatic lineage *in vivo*, but not to germline lineage. In contrast, when we used ES cell culture medium containing 5,000 IU/ml LIF and 2i, established NZB ES cells showed both somatic and germline competency. Our findings suggest that factors required for commitment to germline lineage are independent of those for somatic lineage, and that both high concentration of LIF and 2i are determinants for authentic pluripotency in NZB ES cells. Moreover, to our knowledge, this is the first report of germline-competent NZB ES cells. Because NZB mice display a number of autoimmune abnormalities including hemolytic anemia, elevated levels of im-

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munoglobulin, anti-DNA antibodies, anti-thymocyte antibodies, and circulating immune complexes causing glomerulonephritis, this ES cell line will provide a valuable experimental system for studying the basis of human autoimmune diseases.

Poster Board Number: T-3037

GROWTH AND MAINTENANCE OF EMBRYONIC STEM CELLS IN 3-D CULTURE

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Biocompatible and biodegradable scaffolds have promising applications in tissue engineering and regenerative medicine. They may provide a suitable microenvironment or niche that could stimulate and promote stem cell proliferation, differentiation, and integration *in situ*. The goal of this research is to develop 3-D culturing systems that mimic *in vivo* growth, differentiation and development for tissue regeneration and repair. In this report, we describe thiol-functionalized dextran (Dex-SH) hydrogel scaffold formation via Michael type addition using poly (ethylene glycol) tetra-acrylate (PEG-4-Acr). When Dex-SH and PEG-4-Acr are mixed under aerobic conditions, they self-assemble into a scaffold, which is compatible with embryonic stem cell (ESC) growth. The scaffold was degraded over a period of time in an ESC concentration dependent manner. Scaffold seeded with $2X10^6$ cells/ml degraded slowly compared with the scaffold seeded with $4X10^6$ cells/ml. Seeded ESCs show prolonged growth and remain viable while maintaining self-renewal state. Scaffold cultured ESCs expressed high levels of the pluripotency markers Oct-4, Sox2, and Nanog, in comparison to control ESCs plated by traditional 2-D culture. The self-assembling scaffold provided the necessary support for the growth of ESCs and maintained undifferentiated for several weeks without passaging. The results showed that 3-D ESCs grown in the hydrogel remained pluripotent after 22 days, as judged by their potential for differentiation into osteogenic, myogenic and neural cell types which expressed cell-specific markers, including collagen type1, myogenin, and nestin, respectively. These results suggest that 3-D growth of ESCs in a self-assembled scaffold provides an *in vitro* niche that promotes proliferation while maintaining the self-renewal and pluripotency of ESCs.

Poster Board Number: T-3038

TET1 PROTEIN DEGRADATION SETS THE STAGE FOR THE TRANSITION OF NAÏVE-TO-PRIMED PLURIPOTENCY

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Recent studies have indicated that Tet-family proteins (Tet1/2/3) catalyze the conversion of 5-methylcytosine to 5-hydroxymethylcytocine which can eventually lead to DNA demethylation. Among the Tet proteins, Tet1 in particular has been proposed to be important for the self-renewal of mouse embryonic stem cells (mESCs). On the other hand, the full pluripotency of Tet1-knockout mESCs was confirmed by tetraploid complementation assay and therefore, Tet1 seems to be dispensable for maintaining pluripotency during development. As Tet2, a close Tet1-homolog, is also expressed in mESCs, a single loss of Tet1 may have been compensated by residual Tet2-activities in this case. As a result, the precise roles of Tet proteins in the pluripotency and self-renewal of mESCs remain elusive. Furthermore, the role of Tet proteins in human pluripotent stem cells (hPSCs) is totally unexplored. We first analyzed Tet1 expression profile during the early differentiation of mESCs. As reported previously, Tet1 was abundantly expressed in the undifferentiated mESCs both at the transcript as well as protein levels. Curiously, when these mESCs were induced to differentiate toward an epiblast-like cell (EpiLC) state using bFGF and ActivinA, we started to find discordance between the transcript and the protein level. While Tet1 mRNA level was transiently up-regulated during the process, protein level went significantly down. We attributed this discordance to a ubiquitin-proteasome-mediated protein degradation pathway, as the proteasome inhibitor MG-132 reversed this protein down-regulation. As MG-132 treatment had little effect on Tet1 protein level at the naïve undifferentiated state, we assumed that Tet1 protein degradation might be a developmentally regulated process programmed to occur during the transition from the inner cell mass to the epiblast stage. We then asked whether TET1 instability is also observed in hPSC known to be phenotypically akin to the primed EpiLC. Although TET1 protein is hardly detected in hiPSCs despite of the relatively abundant transcript level, treatment with MG-132 dramatically increased TET1 protein level within the cell nuclei of various hiPSC clones, supporting our assumption that a primed PSC cannot stabilize nuclear TET1 protein as much as a naïve PSC. To examine this possibility further, we sought to assess whether TET1 protein stabilization in hPSCs induces a conversion to a naïve pluripotent state. WNT/β-catenin signaling pathway has divergent roles in naïve and primed pluripotency. It operates for self-renewal to mESCs but induces mesendodermal differentiation to hPSCs. Naïve mESCs can self-renew at the ground state (in the presence of MEK inhibitor and GSK3β inhibitor; 2i) and therefore with potent WNT/β-catenin signaling, but primed hPSCs fail to do so and swiftly differentiate even with a single inhibition (MEK or GSK3β). We found that hiPSCs over-expressing a stabilized nuclear form of TET1 exhibit differentiation resistance against the 2i condition over 10 passages and kept self-renewing. This observation indicates that TET protein activities determine whether a stem cell self-renew or differentiate upon a certain extracellular stimulus. Altogether, our results are suggestive for a primary role for Tet protein degradation in initiating an early event during mammalian development, the naïve-to-primed transition.

Poster Board Number: T-3039

NCOA3 BRIDGES NUCLEAR RECEPTORS, EPIGENETIC REGULATORS, AND GSK3 TO REGULATE NANOG AND PLURIPOTENCY

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Nuclear receptors, including Esrrb, Dax1 and Nr5a2, have been shown to be involved in pluripotency maintenance. Yet, the role of their co-activators in embryonic stem cells (ESCs) remains unexplored. Here, we demonstrated that Ncoa3, but not Ncoa1 or Ncoa2, is essential for pluripotency maintenance. Knockdown of Ncoa3 not only compromises the expression of pluripotency markers, but also impairs in vitro and *in vivo* differentiation potential of ESCs. Ncoa3 binds to the Nanog promoter, and recruits the histone acetyltransferase CBP and the histone arginine methyltransferase CARM1 to activate Nanog expression. Moreover, GSK3 signaling

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down-regulates Ncoa3 protein level to suppress Nanog expression. Thus, Ncoa3 not only contributes to self-renewal by activating Nanog, but also facilitates ESC differentiation as a break point to disrupt the core transcriptional circuitry of pluripotency.

Poster Board Number: T-3040

MMTR FUNCTIONS AS A MOLECULAR RHEOSTAT IN FATE DECISION OF THE PLURIPOTENT ES CELLS BY MEANS OF ITS MULTI-FACETED TRANSCRIPTIONAL REGULATORY ROLES

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Dnmt1-associated protein 1 (DMAP1) was originally identified a DNMT1-interacting molecule and was implicated in gene regulation through modification of chromatin. Recent studies have also revealed that DMAP1 is a core component of the ATP-dependent chromatin-remodeling complexes NuA4/Tip60 HAT and Swr1/SRCAP. MAT1-mediated transcriptional repressor (MMTR) was independently isolated and characterized from mouse embryonic stem (ES) cells as a novel clone and found it to be identical to DMAP1. MMTR is not only a key component of RNA Pol II-mediated gene expression that interacts with HDAC1 and modulates of TFIIH kinase activity via MAT1, but involved in the regulation of cell cycle progression as an intrinsic negative regulator of CAK that regulates mitotic cell cycle progression. It is noted that TFIIH-mediated transcriptional repression activity of MMTR requires intact MMTR whereas CAK-mediated cell cycle regulatory activity of MMTR requires only MAT1-interacting C-terminal half region of MMTR. Thus, MMTR is suggested to play roles in transcription, cellular response to DNA damage, and cell cycle control by interactions with proteins in the different contexts. Here we have sought to dissect the functional roles of MMTR in ES cell self-renewal and pluripotency. We find that the proper expression level of MMTR is crucial for maintenance of ES cell identity. Comparing to wild type cells, ES cell lines with MMTR knockdown or overexpression maintained self-renewal activity with the reduced cell proliferation rate. Embryoid bodies of both these cell lines can undergo the initial steps of differentiation, but seem to be severely compromised in the ability to become committed to a lineage. Indeed, both cell lines could not maintain pluripotency in teratoma assays. Furthermore, we find that N-terminal half-mediated MMTR function is more important than TFIIH-mediated transcriptional repression activity or CAK-mediated cell cycle regulatory activity for maintenance of ES cell identity. Finally, we find that perturbation of the proper MMTR expression level leads to global genetic imbalance at early differentiation stage causing deregulation of pleiotropic biological functions and MMTR exerts both transcriptional activating and repressing function in target gene expression as a component of different protein complexes, including the NuA4/Tip60 HAT complex. Thus, MMTR functions as a molecular rheostat in fate decision of the pluripotent ES cells by means of its multi-faceted transcriptional regulatory roles.

Poster Board Number: T-3041

STAT3 REGULATES OCT4 AND IS ESSENTIAL FOR THE PREIMPLANTATION EMBRYO DEVELOPMENT

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Many previous studies have shown that STAT3 plays important roles in pluripotency maintenance of mouse ES cells. It was also observed that STAT3 knockout mice are embryonic lethal at day 6.5 of development. However, the molecular mechanism of STAT3 involvement in preimplantation embryo development and whether it has a role in regulating the Oct4/Nanog network during the early embryogenesis is not clear. We provide key findings suggesting an essential role of a STAT3/Oct4 axis in the preimplantation embryo and in maintenance of embryonic stem cell pluripotency. Surprisingly we have found STAT3 becomes tyrosine phosphorylated and translocate into nuleus in the 4-cell stage embryo., indicating STAT3 may have a role in this early embrogenesis. We find that STAT3 directly binds to the distal enhancer of, and modulates, Oct4 expression in both ES and iPS cells, reconfirming an important role of STAT3 in maintenance of stem cell pluripotency. Using either maternal or zygotic STAT3 knock out mice, we have established that the STAT3/Oct4 axis is essential for early embryogenesis Taken together, our study provides critical insight information about the role of STAT3 in preimplantation embryo development.

Poster Board Number: T-3042

GENERATION OF EMBRYONIC STEM CELL LINES FROM SOMATIC CELL NUCLEAR TRANSFER BOVINE EMBRYOS IN KOREAN BEEF CATTLE, HANWOO

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Autologous embryonic stem cells derived from somatic cell nuclear transfer embryos (SCNT-ESC) are still valuable in animals for many research applications such as conservation of endangered species and production of transgenic animals. Moreover, SCNT-ESC studies in large animal species like cattle can be a pre-clinical model for patient-specific stem cell therapy in human. In the present study, we established and maintained the SCNT-ESC lines of traditional Korean beef cattle species called HanWoo (Bos taurus coreanae), which is one of the most important livestock in Korean peninsula. Reconstructed embryos by SCNT were cultured *in vitro* for 7 days following electric fusion and activation to obtain blastocyst stage embryos. Each SCNT blastocyst were placed individually on the feeder layer with 3i stem cell culture medium consisting of an equal volume of DMEM/F12 glutamax and Neurobasal medium with 1% (v/v) N2 and 2% (v/v) B27 supplements, plus three inhibitors (3i), 0.8 mM PD184352 (MEK1/2 inhibitor), 2 mM SU5402 (FGF receptor inhibitor) and 3 mM CHIR99021 (GSK3 inhibitor). Primary colonies formed after 2-3 days of culture and the cell colonies were moved onto new fresh feeder layer after 8-9 days of incubation. The intact colonies were routinely passaged every 5-6 days. Eight bovine SCNT-ESC lines have been established from eight different

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SCNT bovine blastocysts. The cells show ESC-like colonies with a distinct boundary and are positive to alkaline phosphatase staining. Immunofluorescence and RT-PCR analyses also confirm their characteristic of pluripotency as Oct4, Sox2, Nanog and stage-specific embryonic antigen-4 is positive. All bovine SCNT-ESC lines are being maintained in culture over 50 passages for more than 270 days. The cells show normal karyotypes consisting of 60 chromosomes (58XX) at passage 50. Embryoid bodies (EBs) were formed by suspension culture to analyze *in vitro* differentiation capability. The marker genes representing the differentiation into three germ layers such as ectodermal: beta-3-Tubulin, Nestin and Vimentin, endodermal: Somatostatin and Gata6, mesodermal: Connexin40 and BMP4 are expressed in EBs derived from bovine SCNT-ESC. This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST; Grant number 2011-0027807) and Technology Development Program for Agriculture and Forestry, Ministry for Agriculture, Forestry and Fisheries (MAFF; Grant numbers 109020-3 and 111160-4), Korea.

Embryonic Stem Cell Clinical Application

Poster Board Number: T-3043

A NOVEL XENO-FREE DEFINED CONDITION FOR CULTURE OF HUMAN EMBRYONIC STEM CELLS AND HUMAN INDUCED PLURIPOTENT STEM CELLS WITH NOVEL HUMAN FEEDER LAYERS

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Pluripotent human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) have the capacity to differentiate into all of the somatic cell types and therefore hold great promise for regenerative medicine. One key issue that needs to be addressed in guiding pluripotent stem cell technology from "bench" towards "bedside" is developing defined cell culture systems for making large banks of cells in cell therapy compliant manner. Here, we have developed a xeno-free culture system for hESCs/hiPSCs and derived a xeno-free cell line of hESC. To completely avoid exposure of hESCs to culture system with animal products, we developed a xeno-free cultivation system for new hESC lines (named as SEES1-4) based on human mesenchymal stromal (HMS) feeders with xeno-free medium. HMS cells were isolated from human dermal tissue samples collected from juvenile donors undergoing surgical procedures of polydactyly with parental written consent of donors and approved by the Institutional Review Board of the NCCHD. To derive and expand HMS feeder layers in xeno-free conditions, we used StemPro MSC SFM Xeno-Free (XF) medium supplemented with StemPro LipoMax Defined XF Lipid Supplement on culture dishes coated with xeno-free matrix Cellstart. We developed a xeno-free culture conditions with the medium for hESCs/hiPSCs that consisted of a KnockOut (KO)-DMEM base supplemented with 15% KO-Serum Replacement XF, amino acids, vitamin C, and several growth factors. All components of the medium were synthetic, recombinant, or of human origin. Three hESC lines (SEES1-3) and 6 hiPSC lines have been maintained and tested their pluripotency over 30 passages in the xeno-free medium on the HMS feeder layer. The cell lines have been maintained by mechanical passaging at early passaging and by recombinant enzyme such as TrypLE Select. In addition, the hESCs and hiPSCs were frozen using a serum-free cryoprotectant. The cell lines exhibit normal hESC characteristics including pluripotency markers, multiple differentiation characteristics as determined by embryoid body analysis and teratoma formation, and are karyotypically normal. Finally, we derived a new hESC line (SEES4) completely in the described xeno-free condition using laser-mediated ablation. We describe a comprehensive characterization of SEES cell lines for retaining hESC-specific features, including an unlimited and undifferentiated proliferation capacity, marker gene expression, stable karyotype, and three germ layers differentiation *in vitro* and *in vivo*. In addition, we observed the expression of nonhuman N-glycolylneuraminic acid (Neu5GC) by HPLC analysis, which was xenoantigenic components of contamination in hESCs cultures, was not detected in xeno-free hESC line. In conclusion, this culture system proved stable maintenance of self-renewal and pluripotency of hiPSCs and newly established hESCs. It will promote the progression toward clinical-grade hESC culture that is critical for developing cell therapy/regenerative medicine paradigms.

Poster Board Number: T-3044

HLA AND ABO GENOTYPES OF EIGHT TAIWANESE HUMAN ES CELL LINES

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Human embryonic stem cells (hESCs) provide a potentially unlimited source for various cell therapies because they can propagate indefinitely *in vitro* and they are pluripotent, which means they can differentiate into all different kinds of tissues of a man. However, the first obstacle encountered in the human ES cell regenerative medicine applications is the histocompatibility problem. Detailed characterization of the blood-histotype of the human ES cells is thus vital to the construction of a human ES cell bank. Here we perform the high-resolution sequence based typing (SBT) of HLA-A,-B,-C,-DQ and -DR loci and acquire complete typing results of eight hESC lines in Taiwan. In our survey, there are several hESC lines that are predicted serologically homozygous at single or double HLA loci: one is homozygous for HLA-A, three are homozygous for HLA-C; one is homozygous for both HLA-C and HLA-DQ loci and one is homozygous for both HLA-A and HLA-C loci. Three of these hESC lines are A blood type (A/A or A/O), three are type O and two are type B (B/B). These documented histotypes of human embryonic stem cell lines as a whole not only provide a detailed reference database for their use in cell transplantation, but also helps to identify their drug testing potentials of the banked human ES cells.

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Poster Board Number: T-3045

BCL-XL OVER-EXPRESSION CONFERS A SELECTIVE GROWTH ADVANTAGE TO HESCS

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During long-term culture hESCs often accumulate chromosomal abnormalities. These tend not to be random and commonly reflect those observed in embryonal carcinoma cell lines. This raises concerns for their use in clinical application and calls for routine karyotype analysis. While standard techniques are able to identify large-scale abnormalities (> ~5MB) molecular analysis is required for detection of small-scale copy number variants (CNVs). A large-scale screen of an ethnically diverse pool of early and late passage hESC lines identified a gain of a region of chromosome 20 amplified in >20% of karyotypically normal hESC lines, predominantly in late passage. This region (20q11.21) has also been found to be over represented in several cancer tissues. The minimal amplicon identified through the screen contains 13 genes, 3 of which are expressed in hESCs: BCL2L1, ID2 and HM13. By performing competition assays we observe that cells expressing elevated levels of Bcl-xL (the dominant isoform of BCL2L1 expressed in hESCs) have a growth advantage over control cells. Shortly after single-cell seeding control cells appear to go into crisis, displaying high levels of apoptosis. Over-expression of Bcl-xL provides protection against this stress-induced cell death and other stresses placed upon cells through general culture. Bcl-xL over-expressing cells are also more resistant to chemical insults when applying agents that activate apoptosis through either DNA damage or cell cycle arrest. This implies that Bcl-xL high expressing cells are more robust in evading cellular protective measures, which maybe undesirable for their use in clinical application. The results suggest that amplification of this region should be screened in addition to standard karyotype analysis.

Poster Board Number: T-3046

ALS DISEASE MODELING USING MOUSE AND HUMAN EMBRYONIC STEM CELLS

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Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease, which is characterized by the loss of upper and lower motor neurons in the brain and spinal cord. To date there is only limited insight in the disease mechanisms and only one drug, Riluzole, is currently FDA approved as a treatment for ALS. Drug discovery has been hampered by the limited amount of animal models and the inability to culture affected cells from patients. Here, we are using mouse and human embryonic stem cells (ESCs) to model ALS. Co-culture of ESC derived motor neurons and primary mouse glia of different genotypes show a non-cell autonomous effect of glia on the survival of motor neurons. This effect is motor neuron specific and is mediated through secreted diffusible factor(s). Using our *in vitro* co-culture system we have identified compounds that inhibit this neurotoxic effect, most of which are targeting inflammatory pathways. Exploring these pathways will lead to a better understanding of disease mechanisms and these compounds could potentially be used as a new ALS therapeutic in the future. We have carried out clinical trials in mice to confirm the protective effect on motor neurons of our most promising candidates. Together, our results propose a model in which ALS is a complex non-cell autonomous disease in which inflammation, glutamate excitoxicity and oxidative toxicity are involved.

Poster Board Number: T-3047

ENABLEMENT OF ALLOGENEIC STEM CELL THERAPIES VIA RADIATION-FREE CONDITIONING INDUCED MIXED CHIMERISM

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Current strategies to combat graft rejection of allotransplants are based on lifelong immunosuppressive regimes, which lead to generalised immunodeficiency and high levels of morbidity, including opportunistic infections and malignancy. A significant problem that will need to be overcome for the successful application of stem cell derived therapeutics. A logical solution to prolonged global immunosuppression is to induce graft-specific tolerance, allowing long-term acceptance of the graft, whilst maintaining immunocompetence. This can be routinely achieved through hematopoietic stem cell (HSC) transplants (HSCT), where graft-matched HSCs re-educate the host's immune system to be tolerant to both host and donor tissue. However current regimes to condition the bone marrow (BM) to receive HSCT involve highly toxic doses of irradiation. Furthermore, successful T cell chimerism, the key to long-term graft acceptance, is dependent on a functional thymus. Paradoxically the thymus undergoes profound atrophy with age, significantly reducing T cell output. Thus, we have been developing a radiation-free protocol to induce tolerance to fully MHC-mismatched allografts and extending this to the aged setting. We show that conditioning with low doses of the anti-neoplastic drug busulfan, in combination with T cell-depleting antibodies and transient immunosuppression, can generate robust levels of mixed chimerism and long-term acceptance of skin grafts. Our laboratory has previously shown that endocrine-based strategies can reverse age-dependent thymic atrophy and enhance immune recovery following HSCT in both mice and humans. Combining a reduced-intensity conditioning regime with strategies for boosting thymopoiesis may aid in the successful translation of this technology to the clinic, to enable long term, drug-free acceptance of stem cell grafts.

Poster Board Number: T-3048

TAU PATHOLOGY FOCUSED DRUG DISCOVERY USING IPSCS IN ALZHEIMER'S DISEASE

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Alzheimer's disease (AD) is the most common neurodegenerative disease and the 6th leading cause of death in the U.S. The histopathological hallmarks of the disease are extracellular amyloid-beta protein (Aβ) plaques and intracellular aggregates of tau protein, with the 2 acting in concert to destroy neurons, leading to a decline in memory function. Multi-transgenic mouse models are commonly used to mimic human disease and while these models have helped our understanding of AD, they have been less successful in identifying new treatments. To address this issue, we at iPierian use human induced pluripotent stem cells (iPSCs) from patients and healthy volunteers to model AD with the goal to identify disease-modifying drugs. In particular, we are interested in the reduction of pathological hyperphosphorylation of tau protein. Due to the already near maximal Aβ burden at the time of AD diagnosis, stop-

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ping the spread of tau pathology may be a more successful strategy when compared to Aβ reducing therapies. We collected skin fibroblasts from over 50 AD patients and controls, including familial presenilin mutants (PSEN1 and PSEN2) and generated iPSC lines from 18 individuals by retroviral transduction. Upon directed neuronal differentiation of these iPSCs we observed the stage specific appearance of genes known to regulate neuronal development and cortical maturation. The emergence of cortical layer specific genes marked the onset of neuronal maturation, and together with a typical neuronal morphology and patch-clamp electrophysiology, indicated a maturation stage suitable for disease modeling. We first characterized the secretion of the amyloid precursor protein cleavage products Aβ40 and 42. Normally, the Aβ40/42 ratio is 10:1, but in familial AD cases (PSEN1 and PSEN2) this ratio is skewed in favor of Aβ42 (4:1). Elevated Aβ42/40 ratio was confirmed in supernatants from our neuronal cultures. We then tested the hypothesis that an elevated Aβ42 load would cause additional pathologies, in particular hyperphosphorylation of tau protein. We were excited to observe that iPSC derived cortical neurons faithfully recapitulated the disease phenotype by exhibiting increased levels (~40%) of phosphorylation at two disease specific tau epitopes when compared to healthy controls. Furthermore this differential was reduced by γ- and β-secretase inhibitors, demonstrating Aβ dependency of tau phosphorylation in these cultures. Currently, we are using this system to validate existing compounds and to identify novel modulators of tau pathology that reduce downstream functional deficits of neuronal health. Functional assays under investigation at iPierian include an assessment of synaptic health, electrophysiological parameters, as well as neuronal survival and process length. Importantly, in addition to studying neurons in isolation, iPSC technology allows us to apply a 'systems approach' to AD, combining patient derived neurons with patient derived astrocytes and microglia. We have successfully established neuron-glia cocultures and are using these culture systems to study cell non-autonomous pathologies of AD. For example, the identification of secreted factors from non-neuronal cells, including pro-inflammatory cytokines, allows us to pursue multiple avenues for therapeutic intervention In summary we have established a novel multi-cell type *in vitro* model system of Alzheimer's disease which we are using to explore new treatment options and to better existing ones.

Poster Board Number: T-3049

GENERATION OF FLUORESCENCE-TAGGED HUMAN EMBRYONIC STEM CELLS BY PIGGYBAC TRANSPOSON SYSTEM

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Genetic modification of human embryonic stem cells (hESCs) is an important tool for gene function research, cell purification and cell tracing after transplantation therapy. To establish transgenic hESCs with fluorescence reporter, we use the piggybac transposon system to generate permanent and reversible gene modification on a Taiwan hESC, TW1. In this transposon system, the piggybac transposase was optimized by mammalian codon usage to increase protein expression efficiency in hESCs. In order to get higher genome integration efficiency, piggybac transposase was driven by CAG promoter. The fluorescence reporter gene was driven by the PGK promoter. The stable transgenic TW1 cells were purified with fluorescence-activated cell sorting and selected with G418 for two weeks. After genome integration, the ratio of GFP-positive cells was about 70%. To eliminate the remaining GFP-negative cells, cells were sorted again and the GFP-positive cell content became over 90%. After FACS purification, we culture these cells for at least five passages without G418-selection. More than 93% of cells were still GFP-positive and the pluripotent marker was persistently in these cells. These transgenic cells were potent to differentiate into rosettes-like neural precursor cells without fluorescence-attenuation. Based on these approaches, researchers can trace this transplanted cell *in vivo* by the GFP fluorescence. Other neuron specific reporter will be applied with this transposon-mediated transgenic system for gene function research, harvesting ES-derived specific neuron lineages and further regeneration researches.

Embryonic Stem Cell Differentiation

Poster Board Number: T-3051

INDUCTION OF BONE MARROW MESENCHYMAL STEM CELLS (BMMSC) THROUGH WHOLE TRANSCRIPTOME MRNA TRANSFER

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Background: Embryonic stem cell (ESC) differentiation and somatic cell reprogramming play an essential role in regenerative medicine and cell therapy. Despite the magnitude of relevant research completed thus far, problems remain in the required amount of resources and the risk associated with genomic alteration. To avoid genomic alteration, recent reports have demonstrated the possibility of utilizing RNAs for such purposes. In our current study, we investigated the feasibility of whole transcriptome mRNA transfer as a method to induce BMMSC from either ESC or somatic cells. Methods: Mouse ESC (mESC) cell line (E14) was maintained in a standard feeder-free condition. Mouse proximal tubule cells (PTC) and BMMSC were isolated from adult mice, and maintained in DMEM/F12 medium with 10% FBS or in αMEM containing 10% MSC-defined FBS (MSC medium), respectively. Mouse BMMSC were transfected with lentivirus carrying EGFP gene (GFP+MSC). mRNA purified from GFP+MSC was transfected into both PTC and mESC under the presence of anti-apoptotic and anti-inflammatory factors; ROCK inhibitor (Y-27632), anti-mouse IL-6, and B18R. Phenotype changes of the transfected cells were characterized after the last transfection. Results: Transfection conditions were optimized for cell viability and transfection efficiency. Although the mRNA mixture purified from cells had low cytotoxicity, the addition of anti-apoptotic and anti-inflammatory factors increased cell viability throughout the mRNA transfection process. By using EGFP signals, we found that the transfection efficiency could be maximized by transfecting mRNA at 1.25 μg/cm2, repeated twice at 12 hour interval. Under these conditions, the levels of mRNA content in the transfected cells were similar to those in the cells from which mRNA were originally extracted. Ongoing studies are underway to characterize the phenotypic changes of the transfected cells, including morphology, gene expression profiles and the ability to further differentiate into osteocyte chondrocyte and adipocyte. Conclusions: This study indicates the potential use of whole transcriptome mRNA transfer as a method to induce BMMSC from mouse ESC and somatic cells.

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Poster Board Number: T-3052

A NOVEL METHOD OF INDUCING GABAERGIC NEURONS THROUGH PDGFRA+ CELLS DERIVED FROM MOUSE EMBRYONIC STEM CELLS

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Background: Successful derivations of specific neuronal and glial cells from embryonic stem cells (ESC) have enormous potential for cell therapies and regenerative medicine. However, the low efficiency, the complexity of induction methods, and the need for purification all represent major obstacles. Takashima et al. induced PDGFRα+ cells from mESC through neuroepithelial cells, and showed that the PDGFRα+ cells contain embryonic mesenchymal stem cells (MSC) (Cell, 2007). In our current study, we found that PDGFRα+ cells induced by the same method can also serve as a useful source from which to differentiate into GABAergic (γ-aminobutyric-acid) neuronal cells. Methods: We differentiated mouse ESC (mESC) on collagen IV-coated plates in αMEM containing 10% MSC-defined FBS with limited exposure to retinoic acid during Days 2-5, and PDGFRα+ cells were sorted on Day 9. PDGFRα+ cells were maintained and proliferated in αMEM containing 10% MSC-defined FBS and ROCK inhibitor (Y-27632). To direct further differentiation, PDGFRα+ cells were cultured for up to 12 days in αMEM supplemented with different combinations of factors: B27, FBS, VEGF, NaN3, bFGF, PDGF-BB, TSA, EGF, Heparin, Thrombin, dexamethasone, β-mercaptoethanol, and Y27632. Results: After 6 days of induction with B27 (or FBS), VEGF, NaN3, bFGF, and Y27632, we detected remarkable expression of GABAergic neuronal markers, such as Dlx2, GAD67, and Pax2 by real time RT-PCR, which reached levels comparable to that of the embryonic brain. We also observed almost 100% GABA- and GAD67- positive cells based on immunostaining. Moreover, the differentiation ability of PDGFRα+ cells was maintained even after more than 10 passages and cryopreservation. Conclusions: This study presents a simple and highly efficient method of inducing GABAergic neurons in only 6 days. Together with its ease of maintenance *in vitro*, PDGFRα+ cells derived from mESC thus prove to be a useful source from which to induce GABAergic neurons.

Poster Board Number: T-3053

CORRELATING EFFECTS OF GEL MICROSTRUCTURAL FEATURES WITH SPECIFIC DIFFERENTIATION PATTERNING OF MOUSE EMBRYONIC STEM CELLS

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Embryonic stem cells (ESCs) have the potential to be used in many applications due to their ability to differentiate into cells of any of the three germ layers (ectoderm, endoderm, and mesoderm). There are several different routes by which ESCs can be guided to differentiate into specific germ layers, including genetic manipulation, chemical cues, and mechanical stimuli. With the latter, it has been observed that substrate stiffness plays a significant role in governing phenotype specific differentiation. In our previous study we investigated the effect of mechanical stiffness of fibrin on ESC differentiation. Our results indicated that while ectoderm and mesoderm germ layers responded weakly to the change in fibrin substrate stiffness in the chosen range (2 - 250KPa), endoderm markers were strongly responsive, with softer substrates up-regulating endoderm specific markers. Although cells respond differently to substrates of varying stiffness, it was also observed that gels with the same stiffness but fabricated under different conditions lead to different differentiation patterning. Therefore, this macroscopic property is perhaps not what the cell truly experiences; modification of fabrication conditions changes the gel microstructure, which in turn changes the stiffness, and these micro-characteristics directly interact with the cells. However, it is not clear what specific microstructural features are the most influential in inducing cellular differentiation, and how they affect ESC behavior. In the current work, we investigate the effect of microstructural features of fibrin gels on the differentiation of mouse ESCs. Twelve different fibrin gels were fabricated by varying the fibrinogen concentration and fibrinogen to thrombin crosslinking ratio. The fibrin gels were used to induce ESC differentiation employing both 2D and 3D cultures. After the differentiation protocol the ESCs were analyzed for phenotypic commitment by performing qRT-PCR for the specific germ layer markers. Each of the 12 different fibrin gels was imaged with scanning electron microscopy. Microstructural features of each of these gels were quantified using an image analysis tool for the characterization of fibrous scaffolds. Specific features which were characterized include fiber diameter, node density, and pore size, and comparison of these attributes along with principal component analysis led to a subset of features which vary most across gel conditions. These gel microstructural features were then correlated with the ESC differentiation patterning using regression analysis. The gels are heterogeneous in nature, and therefore a Monte Carlo sampling technique on the microstructural features was used for regression to account for the variability of the system. Stepwise backward elimination regression was used to reduce the correlation model to include only the most influential parameters. These results are also compared to the correlation of gel stiffness, determined by both by rheological measurements and atomic force microscopy, to phenotype commitment. This analysis reveals the sensitivity of cellular phenotype commitment and differentiation patterning to each of the microstructural features. Moreover, such information can be used to help guide the design of scaffolds with specific properties for tissue engineering applications.

Poster Board Number: T-3054

POPULATION BASED MODEL OF HUMAN EMBRYONIC STEM CELL DIFFERENTIATION DURING ENDODERM INDUCTION

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Understanding the dynamics of differentiating embryonic stem cells (ESC) is essential in the efficient derivation of mature, functional cell types from pluripotent progenitors. Although the theory of differentiation to primary germs layers is known, a mechanistic understanding of the process is still lacking.In this project we are using a population based modeling approach to understand the mechanism of endoderm induction in human ESC . Endoderm induction is performed under two different conditions: Activin A and Activin A supplemented with FGF2 and BMP4. The differentiating cell population is analyzed each day for proliferation, apoptosis, and protein expression. Flow cytometry is used to track the dynamics of expression of two proteins which are indicative of endoderm: Sox17 and CXCR4. In order to elucidate the mechanism of differentiation, a stochastic population based mathematical model was developed based on an earlier model reported for the hematopoietic stem cell system.The simulation is initiated with a population of un-

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differentiated cells, wherefrom it evolves in time by assigning each cell a propensity to proliferate, apoptose and differentiate using certain user defined rules. The propensity of each cell to differentiate is tracked with time, and upon reaching a certain threshold the cell is considered committed to a particular lineage. Because it is likely that a parameter hyper-space will better describe the experimental data than a single parameter combination, an ensemble parameter estimation was performed by randomly generating values from the hyper-space of the sensitive parameters and taking those parameter sets which give errors less than a tolerance threshold. It is observed that the simulated dynamics are quite sensitive to the mechanism of differentiation prescribed to the model. A comparison of the agreement of experimental data with model prediction for several competing mechanisms allows identification of the most probable mechanism of endoderm specification of hESCs. Twelve different plausible mechanisms which might describe the observed dynamics were developed, and this ensemble method was performed on each of these mechanisms. For each case, the model ensembles of proliferation, apoptosis and differentiation dynamics are compared with respective experimental data to determine the most probable mechanism. This analysis led to the identification of one mechanism which adequately describes the observed dynamics. The results indicate that hESC commitment to endoderm occurs through an intermediate mesendoderm germ layer which further differentiates into mesoderm and endoderm, and that the proliferation of specific phenotypes is favored over others. Furthermore, our model suggests that CXCR4 is expressed in the mesendoderm and endoderm germ layers, but is not expressed in mesoderm. Comparisons between the two induction conditions indicate that supplementing FGF2 and BMP4 to Activin A enhances the kinetics of differentiation. The model is validated against a totally different set of test data for two different genes which was not used during model training, and shows excellent agreement with the experimental dynamics. The population based model used in this study represents a valid platform for identifying mechanisms governing dynamic biological systems, and can be extended to any system of adult stem cells or to later stages of ESC differentiation to more mature phenotypes.

Poster Board Number: T-3055

DIFFERENTIATION OF HUMAN PLURIPOTENT STEM CELLS TO CD34$^+$ HEMATOPOIETIC PROGENITOR CELLS USING STEMDIFF™ HEMATOPOIETIC KIT

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Human pluripotent stem cells (hPSCs), including embryonic stem cells (hESC) and induced pluripotent stem cells (hiPSC), are characterized by their potential to differentiate into any somatic cell lineage. A diverse set of multi-step directed differentiation protocols have been described to induce differentiation to desired cell types, using a variety of media formulations, in either embryoid body (EB)-based or adherent cell-based cultures. We have developed a simple and efficient culture system for differentiation of hPSCs to hematopoietic progenitor cells. This is an adherent cell-based protocol whereby hPSCs are differentiated directly on a Matrigel™-coated 2D surface, thereby omitting the EB formation stage. Small undifferentiated hPSC aggregates from the H9 hESC or WTS4D1 hiPSC lines were seeded onto Matrigel™-coated 6-well plates in mTeSR™1 maintenance medium, and cultured for 2 days to allow colony attachment and growth. Differentiation was initiated (day 0), by replacing the maintenance medium with 3 mL of STEMdiff™ Hematopoietic Basal Medium supplemented with STEMdiff™ Hematopoietic Supplement A. Cells were cultured in this media for 4 days to allow mesoderm induction. On day 4, the medium was changed to STEMdiff™ Hematopoietic Basal Medium supplemented with STEMdiff™ Hematopoietic Supplement B. Cells were cultured in this medium for a further 9 days, with complete media changes on days 7 and 10. At the end of the 13-day culture period, cells were harvested, counted, and fractions of the population analyzed by flow cytometry for the hematopoietic markers CD34 and CD45, or plated in standard hematopoietic colony-forming cell (CFC) assays using MethoCult™ H4434. Differentiation of H9 hESCs resulted in yields of $1.7 \pm 0.5 \times 10^6$ cells per well (n=10, all values represent mean ± SD), containing $26.0 \pm 8.8\%$ CD34$^+$ cells, and $9.5 \pm 6.6\%$ CD34$^+$CD45$^+$ cells. Moreover, the differentiated H9 cell population contained 219 ± 165 CFCs per 10^5 cells (n=9), or approximately 1 CFC per 450 cells. Similar results were obtained when the STEMdiff™ Hematopoietic Kit was used on WTS4D1 hiPSCs, with yields of $2.63 \pm 0.6 \times 10^6$ cells per well (n=7), containing 29.9 ± 11.1 % CD34$^+$ cells, and 6.9 ± 4.0 % CD34$^+$CD45$^+$ cells. The differentiated population obtained from WTS4D1 cells contained 126 ± 40 CFCs per 10^5 cells or approximately 1 CFC per 788 cells. Overall, the STEMdiff™ Hematopoietic Kit is simple to use and highly efficient for production of hematopoietic cells and progenitors.

Poster Board Number: T-3056

DIFFERENTIATION OF HUMAN INDUCED PLURIPOTENT STEM CELLS TO BEATING CARDIOMYOCYTES USING STEMDIFF™ CARDIOMYOCYTE KIT

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Human pluripotent stem cells (hPSCs), including embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) have two defining functional characteristics: self-renewal divisions to generate pluripotent progeny, and differentiation capacity to any somatic lineage. Here we report the development of the STEMdiff™ Cardiomyocyte Kit, a defined, serum-free system for the efficient differentiation of beating cardiomyocytes from hiPSCs. The system utilizes an embryoid body (EB)-based method of differentiation, wherein EBs are cultured with a specific series of factors designed to mimic *in vivo* developmental cues driving cardiac specification. Using the hiPSC lines WCS-4D1 (provided by Dr. William Stanford, Toronto, Canada) and A13700 (Life Technologies), we harvested cells from mTeSR™1 cultures, and used AggreWell™400 microwell-textured plates to form EBs of 1,000 cells each in STEMdiff™ Cardiomyocyte Basal Medium with a unique Supplement A. STEMdiff™ Cardiomyocyte Basal Medium is a novel serum-free and animal component-free medium, which is used to maintain EBs in this protocol with a series of unique supplements (A through E) used sequentially at defined timepoints from day 0 through day 16. The EBs were cultured within the AggreWell™400 plates for the entire duration of the protocol, and on specific days the culture media was changed carefully to minimize disruption of the EBs in the microwells. Due to differences in individual cell lines' responses to inductive signals, Supplement B, was first titrated on each cell line to determine the optimum amount for maximal cardiac induction, and that amount was then used for subsequent experiments. Using optimized amounts of Supplement B on day 1 and indicated amount of Supplements C through E added on days 4, 8, and 12 respectively for each hiPSC line, beating EBs were observed as early as day 12 of differentiation. By day 16 synchronous beating was ob-

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served in 87% ± 9% of WCS-4D1 EBs (n=7) and 92% ± 5% of A13700 EBs (n=5; values represent mean ± standard deviation). All EBs were also harvested from the AggreWell™400, dissociated into single cell suspensions using Collagenase II, and analyzed by flow cytometry for expression of the intracellular cardiomyocyte marker cardiac troponin T (cTnT). Under these conditions, 23 ± 11% (n=5) and 26 ± 14% (n=3) of WCS-4D1 and A13700 derived cells, respectively, expressed cTnT. Moreover, EBs could be dissociated and resulting cells plated onto Matrigel™ coated dishes where they could be maintained as a beating monolayer for at least 60 days. Cardiomyocytes replated onto Matrigel™ were also fixed and analyzed by immunocytochemistry for cTnT expression, and displayed a characteristic striated staining pattern. Overall, these results demonstrate that the STEMDiff™ Cardiomyocyte Kit can be used to efficiently and reproducibly form beating cardiomyocytes from hiPSCs maintained in mTeSR™1 defined and feeder-independent culture conditions.

Poster Board Number: T-3057

GENERATION AND FUNCTIONAL CHARACTERIZATION OF HEPATOCYTES FROM HUMAN PARTHENOGENETIC STEM CELLS

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Hepatocellular transplantation offers an attractive alternative to orthotopic liver transplant, and *in vitro* cultured pluripotent stem cells could become a great source of hepatocytes. Human parthenogenetic stem cells (hpSC) possess all advantages of embryonic stem cells yet their derivation does not require viable embryo destruction, and does not pose ethical concerns. In addition, hpSC homozygous for HLA antigens can be obtained relatively easily, providing a great histocompatibility advantage during implantation. Previously, we described a three-dimensional (3D) culture system allowing for generation of definitive endoderm at unprecedented homogeneity. We demonstrated the usefulness of this system producing high purity populations of hepatocyte-like cells. Here were optimize, streamline and evaluate our system as a potential source of hepatocytes for cell-based liver therapy. .In order to enable the adequate testing, and possible future therapetic use, we first optimized our culture conditions using larger custom-built culture devices with adequately bigger porous membranes, which allowed to generate several millions of highly pure hepatocytes in a single batch. Careful analysis using flow cytometry, immunostaining and PCR confirmed the desired phenotype of the obtained cells, as evidenced by enzymatic activity of CYP450, ICG uptake, glycogen storing, and demonstrated secretion of hepatic proteins, such as AFP, ALB, AAT1, Plasminogen, Factor IX. To gain further insight into the functional capabilities of the generated hepatocytes we tested them using two animal models. First, we intrasplenicly injected the hpSC-derived hepatocytes into SCID mice with acetaminophen-induced acute liver failure. We observed that our CFSE-labeled cells migrated to the liver, integrated into the liver parenchyma, and acquired hepatic characteristics. Immunohistochemical analysis revealed the presence of the engrafted human, albumin-positive cells. Detectable levels of human liver-specific proteins (hAFP, hAAT1, hALB) in the serum of injected mice were observed at day 30 post transplantation. At day 60, hAFP became undetectable, while relatively high levels of both hAAT1 and hALB were found in the blood. This indicates further differentiation to fully mature hepatocytes in their appropriate endogenous environment of the host liver. In addition, we have treated juvenile Gunn rats with a single intrasplenic injection with hpSC-derived hepatocytes. Overall, the inoculation of tested cells revealed stable clinical remission of both acute liver failure and chronic metabolic disease in experimental animals. Our work demonstrates that pure hepatocytes can be produced from hpSC in reasonable numbers at acceptable cost. Personalized immuno-matching, as well as the ability to undergo terminal maturation *in vivo* make these cells an attractive candidate for cell-based transplantation in regenerative therapy.

Poster Board Number: T-3058

LINEAGE PRIMING AND CONTROL OF ENDODERM DIFFERENTIATION IN HUMAN EMBRYONIC STEM CELLS

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Following prolonged culture *in vitro* human embryonic stem cells become more robust and easier to maintain, displaying higher cloning efficiencies and reduced population doubling times. This phenomenon termed 'culture-adaptation' is also closely associated with cytogenetic changes including partial or whole chromosome duplications and deletions. Interestingly these karyotypic abnormalities are similar to those observed in human embryonal carcinoma cells suggesting that these cytogenetic aberrations may provide a selective advantage over their 'normal' diploid counterparts. Recent evidence suggests that 'culture-adapted' human embryonic stem cells may also be lineage biased when induced to spontaneously differentiate via the formation of embryoid bodies. The 'culture-adapted' cells appeared to be deficient in their ability to differentiate towards an endodermal lineage. To investigate this further we used a late passage, karyotypically abnormal human embryonic stem cell line, which displayed characteristics of 'culture-adaptation' and compared it with its early passage, diploid counterpart. Using whole population and small-scale differentiation protocols we found that 'culture-adapted' cells have a reduced capacity to differentiate towards a definitive endoderm lineage when compared with their early passage counterpart. This is determined by marked decreases in transcript levels of definitive endoderm markers such as Sox17 and FoxA2. We also observed reduced levels of endoderm markers by immunofluorescene and flow cytometry, with the 'culture-adapted' cells displaying up to 70% reduction in Sox17, FoxA2 and CXCR4 levels when compared with lower passage, 'normal' cells. We are investigating the mechanisms underlying this lineage bias to gain insight into the early lineage decisions of differentiating human embryonic stem cells.

Poster Board Number: T-3059

REGULATION PATTERN OF REST IN HUMAN INDUCED PLURIPOTENT STEM CELLS UNDERGOING NEURAL DIFFERENTIATION

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Introduction: The neuronal repressor REST (RE1-silencing transcription factor) is expressed at high levels in embryonic stem cells (ESCs) with a critical role in self-renewal and pluripotency signaling network of these cells. REST is an essential element for brain devel-

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opment and also neuronal differentiation of ESC *in vitro*. Although REST is a well-known regulatory element in embryonic stem cells, but according to our knowledge, it has not been evaluated in neural differentiation of human induced pluripotent stem cells (hiPSCs) and till now. Aims: In this study, we analyzed the expression pattern of REST during the stepwise differentiation of human ESCs and human iPSCs to neural precursor cells (NPCs) and then to mature neural cells (MN). Material and Methods: The quantitative expression of REST in each step was evaluated by real-time RT PCR and the presence of REST was showed by immunofluorescent assay. The epigenetic regulation of this pattern was performed by Chromatin Immunopercipitation (ChIP) assay. Results: q-RT PCR analysis showed that the expression level of REST decreased significantly during differentiation of human pluripotent stem cells (hPSCs) to neural precursor cells, and remained stable until neuronal differentiation. Immunofluorescent data revealed the both nuclear and cytoplasmic presence of REST in hiPSCs as well as NPCs, while in the mature neurons it was only detectable in the cytoplasm. The data from the study of selected epigenetic marks was in accordance with the gene expression results. Conclusion:. The pattern of REST expression during hPSCs neural differentiation in our findings was in accordance with one of the previously reported articles. This study opens a new window for further experiments in this field for gaining an efficient differentiation of neuronal cells.

Poster Board Number: T-3060

HIGH QUALITY RNA ISOLATION AND TRANSCRIPTOME PROFILING OF HUMAN CELLS PURIFIED BY FACS USING INTRACELLULAR MARKERS WITHOUT REPORTERS

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Stem cell differentiation and somatic cell reprogramming typically give rise to highly heterogeneous cell populations, hampering molecular analysis of specific subpopulations. We optimized a method to obtain high-quality, global gene expression profiles of primary human and hESC-derived cells purified by fluorescence-activated cell sorting (FACS) using cytoplasmic markers. A molecular signature was obtained from primary human pancreatic beta-cells FACS-purified from other endocrine cell types. We compared the beta-cell transcriptome to that of insulin-positive cells derived from genetically unmodified hESCs. Differences in signaling and metabolic pathways suggested directed differentiation culture modifications that generated insulin-positive hESC progeny whose gene expression more closely resembled mature human β cells. This strategy holds broad potential for comparing pure cell populations from directed differentiation cultures and native human tissue without the use of reporters.

Poster Board Number: T-3061

EFFICIENT DERIVATION OF VASCULAR SMOOTH MUSCLE CELLS FROM HUMAN EMBRYONIC STEM CELLS

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Developing disease-specific cellular models is critical for understanding the molecular mechanism of the disease and is expected to have a major impact on the development of new therapies and regenerative medicine. Several genetic syndromes (such as Marfan and Loeys-Dietz Syndromes), congenital vascular diseases and hereditary influences are closely associated with vascular smooth muscle (vSMCs) dysfunction in the thoracic aorta (Atherosclerosis, hypertension, aortic aneurysm). A major challenge in vascular disease drug development is the identification of model systems that accurately recapitulate normal and diseased vascular physiology in vitro. Human Embryonic Stem Cells (ES) are pluripotent and can differentiate into all the different cell types of the body. Additionally, ES cells are capable of propagating themselves indefinitely. Because of their plasticity and unlimited capacity to proliferate, ES cells represent a potentially renewable source of vSMCs. We are now in the process of deriving hESC lines carrying the chromosomal mutations responsible for Marfans and Loeyz-Dietz Syndromes. The availability of disease-affected stem cells is the first step towards a vascular disease model. However, the critical next step is to differentiate stem cells into vSMCs, the affected cell type. Currently, vSMC derivation from human embryonic stem cells (hESCs) usually requires serum, the generation of 3D embryoids bodies which does not provide a pure population of vSMCs and is not suitable for drug development. Therefore, we performed a high-throughput screening to determine optimal differentiation conditions and establish a protocol to efficiently generate vSMCs in vitro. Using our well-established stem cell differentiation discovery platform (including robotics and high-content analysis), we screened a targeted library of known regulators of cell differentiation pathways to identify a simple and rapid process to produce vSMCs with high efficiency. Here, we describe a chemically defined monolayer system in which hESCs are initially induced to form cardiovascular progenitors from the mesoderm lineage (Isl1+, Nkx2.5+, SSEA1+). This almost pure, intermediate population is further differentiated toward SMCs (80% SM-MHC+, α-SMA+, Calponin+, Caldesmon and Smoothelin B), which displayed contractile ability in response to a vasoconstrictor (carbachol). This differentiation approach and our ability to produce large amounts of vSMCs from disease-specific hESCs will have broad applications in modelling vascular diseases and is therefore a significant contribution towards a better understanding of disease mechanisms, and it will ultimately to assist in drug discovery and the development of effective treatments.

Poster Board Number: T-3062

REGULATION OF TRANSCRIPTIONAL FACTOR GATA4 BY MIR-200C IN HUMAN EMBRYONIC STEM CELLS

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Human embryonic stem cells (hESCs) can self-renew and differentiate into almost all types of cells of the body. *In vitro*, hESCs can differentiate into embryoid bodies (EBs) that contains three germ layers ectoderm, mesoderm, and endoderm. MicroRNAs (miRNAs) are small non-coding RNAs that govern key cellular processes. miR-200 family plays crucial roles in epithelial-mesenchymal transition (EMT) in cancer cells. EMT process is important for hESC differentiation. However, no studies have been performed to investigate the functional roles of human miR-200 family in hESC renewal and differentiation. In this study, we found that miR-200c expression is most abundant in undifferentiated hESCs among the miR-200 family (miR-200a, miR-200b, miR-200c, miR-141, and miR-429). Furthermore, miR-200c is enriched in undifferentiated hESCs and is downregulated in EBs. The knockdown of miR-200c in hESCs caused cell differentiation and induced GATA binding protein 4 (GATA4) expression. Interestingly, overexpression of miR-200c decreased the efficiency of EB formation and repressed the markers of

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all three lineages (ectoderm, endoderm, and mesoderm). We also found that knockdown of GATA4 in hESCs also decreased EB formation. Of note, miR-200c can directly and specifically target the 3'-untranslated regions of the GATA4 in a luciferase assay. Furthermore, miR-200c-mediated GATA4 expression played an important role in hESC proliferation. Knockdown of miR-200c inhibited cell proliferation which can be partially rescued by the blockage of GATA4. On the other hand, the overexpression of miR-200c increased the expansion of hESCs that can be blocked by the overexpression of GATA4. Taken together, miR-200c-mediated GATA4 regulation coordinates hESC proliferation and differentiation. In undifferentiated state, the miR-200c expression promotes hESC renewal and blocks hESC differentiation. During EB formation, the decreased expression of miR-200c is responsible for GATA4 upregulation and promotes the commitment to EBs. This findings uncover a new mechanism of the undifferentiated and differentiated state of hESCs with a focus on the hESC to EB transition.

Poster Board Number: T-3063

MODELING HUMAN GERM LAYER FORMATION USING HUMAN PLURIPOTENT STEM CELLS

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Human embryonic and induced pluripotent stem cells potentially serve as very powerful tools for studying human development. The early events of development have been well studied in model organisms such as sea urchin and mouse, but analogous events in human are not yet well described at the molecular level, due to both technical and ethical issues. Human embryoid bodies (hEBs) are cell aggregates generated from hPSCs. Human EBs have the potential to differentiate into all three germ layers and have been suggested as a possible model for human development. We have characterized the early stages of hEB differentiation and screened for various markers of germ layer formation. We have devised a method for labeling and purifying a population that appears to mimic the first epithelial-to-mesenchymal transition in the human embryo and that shows characteristics of the early mesendoderm. We have isolated and characterized this population in order to uncover novel germ layer determinants and understand the molecular basis for germ layer formation. We are currently examining the potential of this mesendodermal population to not only model human development but also to develop novel methods for the generation of high purity mesendodermal cell types from hPSCs.

Poster Board Number: T-3064

THE GENERATION OF LUNG PROGENITORS FROM HUMAN ES CELLS

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Directed differentiation of human embryonic stem (hES) cells and induced pluripotent stem (hiPS) cells into midgut and hindgut endoderm derivatives has been successfully established. The generation of anterior foregut lineages (i.e. trachea, lung and pharyngeal organs) proved more challenging, however. Previously, we showed that dual inhibition of transforming growth factor (TGF)-β and bone morphogenic protein (BMP) signaling in definitive endoderm resulted in a highly enriched population of anterior forgut endoderm (AFE) cells. By mimicking *in vivo* sequential lineage specification, we ventralized the AFE and specified a fraction of the cells into FOXA2+Nkx2.1+PAX8- early lung progenitors. Here, we further pursued the differentiation of cells of the respiratory system. By modifying the anteriorization protocol, we increased the efficiency of FOXA2+NKX2.1+PAX8- cell generation at day 15 from 37% to approximately 60%. Our data suggest that at this early point in differentiation, AFE cells are prespecified to a subsequent lung, as opposed to pharyngeal fate ten days later. We next devised conditions that specify proximal vs. distal fates. By day 23, cultures from both proximal and distal conditions consisted of highly structured dense epithelial colonies. 70~100% of the cells were FOXA2+NKX2.1+. Under proximal conditions, 90~100% of the cells were SOX2+. Cells at the periphery and discrete cells within the colony expressed p63, a marker of airway basal cells. Continued culture with addition of maturation components yielded clusters of CC10+ Clara cells, mucin5AC+ goblet cells or mucin2+ cells at d35 within these colonies. Mucin5AC+ cells formed tubular structures. Linear structures composed of acetylated-α tubulin+ ciliated cells occurred at the edge of the colonies. Cultures in matrigel yielded spherical structures lined by p63+ cells that are consistent with tracheospheres obtained from adult tracheal basal cells. Day 35 cultures from 'distal' conditions were also able to differentiate into CC10+, mucin5AC+ or mucin2+ airway lineages. Although distal alveolar epithelial (AE) markers such as SPB and mucin 1 could be detected in the cultures, distal conditions need to be further optimized to block the proximal fates and enrich type I and type II alveolar epithelial cells. Importantly, after seeding onto slices of decellularized lung matrices, the cells lined up along the matrix. Further differentiation and spatial organization of the cells in this model is currently investigated. We conclude that we achieved, for the first time, highly efficient differentiation of hES cells into most lineages of respiratory cells with minimal contamination from other lineages. Importantly, this strategy does not require enrichment of cells committed to a respiratory fate based on reporter gene expression. This work is a major step towards regenerative medicine for lung disease using decellularized human lung matrices seeded with hiPS-derived respiratory cell populations.

Poster Board Number: T-3065

DIRECTED DIFFERENTIATION OF HUMAN PLURIPOTENT STEM CELLS TO HOMOGENEOUS MULTIPOTENT NEURAL PROGENITORS

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Numerous protocols have been published on directing pluripotent or multipotent stem cells to the neuroectodermal lineages. Most of these reports demonstrate the propensity of the cells toward a single neural lineage, either primarily neuronal or glial, but not both. We sought to capture a homogeneous population of multipotent NPCs derived from human ES/iPS cells. As a function of homogeneity, the majority of the starting NPC population should in response to appropriate inductive signals, be directed towards specific cell lineages. For example, in response to neuronal inductive signals, a majority of NPCs should be directed to βIII-tubulin positive neurons. Similarly in response to glial inductive signals, the same starting multipotent neural progenitors should now give rise to mostly glial cells. Using a cocktail of three small molecules that inhibited TGFβ, BMP and GSK3β signaling pathways, human ES/iPS cells could be efficiently differentiated in 7 days to multipotent NPCs that expressed 70% Pax6 positive cells. The protocol was able

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to accommodate variable initial cell densities and was applicable for both ES and iPS cells cultured on either feeder or feeder-free based culture systems. To demonstrate multipotency, NPCs were differentiated into either predominantly neurons or oligodendrocytes. Under neuronal differentiation conditions, approximately 90% of NPCs expressed neuronal markers, βIII-tubulin and MAP2 after 9 days. Alternatively NPCs could be directed toward primarily oligoglial lineages (>50% PDGFRα and GalC) after 14 days of oligodendrocyte differentiation. These results suggest that we have captured a homogeneous population of multipotent NPCs. Homogeneous multipotent NPCs are an effective tool in which to apply existing knowledge to pattern neural cells toward anterior, posterior and mid-brain fates for region specific differentiation.

Poster Board Number: T-3066

EFFICIENT MYOGENIC COMMITMENT OF HESC-DERIVED CELLS ON THE BIOMIMETIC SUBSTRATE REPLICATING MYOBLAST TOPOGRAPHY

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Recent development in stem cell technology has demonstrated human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) as possible cells for cell-based therapy and studies of particular differentiation program. However, spontaneous and heterogeneous differentiation of these cells may limit the potential use. Here, we describe the efficient generation of mesenchymal cell population from both hESCs and iPSCs having non-turmoigenic potentianl and their commitment into myoblast. In skeletal development, increased cell-cell contact plays critical initial steps for myogenic commitment. We demonstrate that iPSCs and hESCs-derived cells can undergo efficient myogenic commitment by topographical cues present in their environment. We have created substrates from biomimetic materials that can replicate the mico- and nanoscale topography of fully differentiated skeletal myoblast. When hESCs and iPSCs-derived mesenchymal cells were cultured on biomimetic pattern, mesenchymal cells followed the underying myoblast pattern. Furthermore, gene expression and cell fusion index showed enhanced myogenic commitment on these substrates. These results demonstrate that myogenic potential of hESCs and iPSCs-derived cells are highly dependent on the micro- and nanoscale topographical cues provide by skeletal myoblast

Poster Board Number: T-3067

NOVEL COMBINATORIAL APPROACHES TO DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS TO HEPATIC-LIKE CELLS.

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Hepatocytes derived from human embryonic stem cells (HESC) have significant potential to provide an unlimited source of cells for evaluation of drug metabolism and toxicity, and for regenerative medicine. To realise this potential an efficient and robust protocol that approximates natural hepatocyte development is required. This project aims to systematically specify and optimise such a protocol using novel combinations of small molecules and growth factors. HESC are directed towards hepatocytes in a stage-specific and time-dependent manner. HESC cultured in monolayers are first exposed to Activin A and varying combinations of small molecules to initiate and direct differentiation towards definitive endoderm. To initiate gene expression leading to hepatoblast formation cells are treated with a combination of growth factors that are released during embryogenesis, supplemented with additional factors that further enhance this process. Combinations of cytokines and growth factors, including OSM and HGF, together with glucocorticoids and insulin, are used at certain time points and specific concentrations to prime the cells to form hepatic-like cells. Finally a range of defined culture conditions are used in order to create niche conditions stimulating further hepatic maturation. We have found that inclusion of certain small molecules at various time points of differentiation has a significantly beneficial impact on generation of hepatic-like cells. The identity of these cells has been confirmed by the analysis of hepatocyte-specific markers including Albumin and ASGR1 using flow cytometry, quantitative RT-PCR and immunocytochemistry and ELISA. Results from this work will be presented to highlight the differences between standard and small molecule-enhanced protocols for differentiation and maturation of HESC derived hepatocytes.

Poster Board Number: T-3068

RAPID AND EASY MONOLAYER NEURAL DIFFERENTIATION OF HUMAN ES CELLS

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Human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) have the potential to differentiate into various cell types including neural cells.Recently, hESC- and hiPSC-derived neural cells have been expected to be applied not only to regenerative medicine, but also to studying pathophysiology of neurological diseases and human neural development as an *in vitro* model. However, previously established neural differentiation methods for human pluripotent stem cells, including our own method, take long time to derive neural cells *in vitro*. Moreover, because of its length of time, it has been difficult to analyze functional properties of hES cell-derived neural cells by transient overexpression or knockdown of specific target genes. Thus, in the present study, we developed a rapid and highly efficient monolayer neural differentiation system, with high transfection efficiency. Because our neural induction method needs to be simple and easy to handle, we optimized culture medium, culture period, and differentiation substrates for neural induction, and established a monolayer culture system which requires only several-times of culture medium change during wholeperiod of the neural induction. Finally, by using our monolayer neural induction method, hESCs could rapidly differentiate into SOX1-positive neural progenitors only in 6 days, and into βIII-tubulin-positive post-mitotic neurons only in 16 days. In addition, by transfecting a siRNA against a neural specofic gene SOX1at the beginning of neural induction, we confirmed high knockdown efficiency at day 3 and day 6 after transfection.Currently, by using this system, we are screening candidate genes, which may be involved in human neural development, identified based on global gene expression analysis of several lines of hESCs and hiPSCs.

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Poster Board Number: T-3069

HUMAN EMBRYONIC STEM CELL DIFFERENTIATION IS CHARACTERIZED BY PROMOTER SWITCHING IN GENES CODING FOR TRANSCRIPTION FACTORS

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Transcription initiation is a key component of gene regulation. Insight into promoter regulation and choice of transcription start sites (TSS) in differentiation using cap analysis gene expression (CAGE) gives high resolution alternate initiation patterns during developmental events and cellular reprogramming. Developmental TSS switching implies promoter utilization changes or 'switches' from one to another during differentiation which provides us with insight into: a) lineage specific regulatory events, b) alterations in the form of the final transcript, c) changes in TF utilization and binding, and thus associated changes in regulatory networks directing cell differentiation. Unprecedented resolution of exact TSS choice provides precise locations for upstream regions and thus yields high-fidelity models of transcription factor (TF) binding events using motif prediction. Further, a genome-wide, multi-expression-state TF binding prediction can be performed across promoters in-silico complementing ChIP-Seq approaches, which may be used for independent validation. We have developed SwitchEngine, a statistical algorithm for detecting TSS switching in CAGE time-course data. It robustly detects switching events that have been manually validated. In this study we present a 41-day time-course experiment in which H9 embryonic stem cells were differentiated to melanocytes. SwitchEngine detected statistically significant promoter switching events in 439 genes in the most differentiated state as compared to the original embryonic stem cells (p-value < 0.05). These show a significant enrichment for transcription factors and other regulatory elements when analyzed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Benjamini-adjusted p-value < 0.001). Of all genes in which switching was detected, 22.5% of them belong to the "regulation of transcription" GO category, implying that TSS switching plays an important part in TFs' role in driving differentiation.

Poster Board Number: T-3070

SMALL MOLECULE-BASED SELECTIVE ENRICHMENT OF CARDIOMYOCYTES FROM HUMAN ESC AND IPS CELLS

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Purpose: Pluripotent stem cell (PSC)-derived cardiomyocytes (CMs) have a great potential for cardiac replacement therapies, in vitro tissue engineering and more reliable safety pharmacology assays. These applications require generation of highly purified, well characterized CMs depleted of other cell types. We and others have previously described genetic methods for lineage enrichment resulting in essentially pure CMs. These methods, however, rely on the genetic manipulation of PSC and the expression of reporter genes which has a number of limitations, particular for therapeutic applications. CM-enrichment based on a lineage-restricted surface marker has also been published, but cell recovery and vitality subsequent to cell sorting procedures such as MACS or FACS is usually low. To overcome above issues we have investigated small molecule-based strategies for CM enrichment from human PSCs. Methods: Human ESCs and iPSCs were differentiated by 2 established, independent methods both requiring ESC aggregation into embryoid bodies (EBs). A small-throughput 6well-based screening platform was established to test small-molecule candidates, optimize compound concentration(s) and timing of supplementation. Differentiation efficiency and CM purity was analyzed by flow cytometry, immunofluorescence analysis and qRT-PCR; electrophysiological assessment was further applied for CM characterization. Furthermore, general proliferative and/or toxic influence of applied small molecules on undifferentiated human PSC was assessed by cell cycle analysis and proliferation assay. Results: We have identified a class of molecules enabling efficient enrichment of CMs from other lineages. Protocol optimization allowed enrichment of at least 80% CM purity. Besides generally supporting CMs enrichment, gene expression and electrophysiological analysis further suggested a potential selection of CMs with an atrial/pacemaker-like phenotype in response to small molecule treatment. The method is robust, was successfully applied to several human PSC lines and was compatible with 2 independent differentiation protocols. Currently, potential mechanisms underlying the observed selectivity are under investigation. Conclusion: To our knowledge this is the first report of small molecule-based enrichment of CMs from human ESC and iPS cells. The adaptation of the method to larger scale CMs production as a next step might provide sufficient cells for their envisioned applications. The technology might not only be utilized for CMs purification and production. Ongoing investigations of the underlying mechanisms will also shed new light on early steps of human cardiomyocyte differentiation, cell survival, proliferation and cell physiology.

Poster Board Number: T-3071

STEPWISE INTERVENTION FOR PROMOTED HEMATOPOIETIC DIFFERENTIATION FROM HUMAN PLURIPOTENT STEM CELLS

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Hematopoietic systems in mouse models have been well characterized based on the defined cell surface markers present on fetal and adult hematopoietic stem cells (HSCs) and their blood derivatives. In humans, by contrast, hematopoietic ontogeny and the subsequent hierarchy have not been determined. Human pluripotent stem cells (ES cells, ESCs and iPS cells, iPSCs) are embryo-type cells and a promising cell source for studying the ontogeny of blood cells within a differentiation system. We previously established an in vitro co-culture method using C3H10T1/2 mesenchymal stromal cells, whereby vascular endothelial growth factor (VEGF) promotes the appearance of CD34+ hematopoietic progenitor cells (HPCs) from human ESCs or iPSCs (Takayama et al., Blood, 2008; Takayama et al., J Exp Med, 2010). Here we demonstrate that optimized in vitro hematopoietic differentiation system can be divided into specific developmental stages that include CD56+CD326- mesodermal progenitors during the early-phase (days 0-4),

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CD34+CD56+CD90+CD105+CD43-KDR- hemangioblasts during the mid-phase (days 5-7), and CD43+ hematopoietic and KDR+ endothelial cells during the late-phase (days 8-10). Using these markers as indicators, we attempted interventions in each phase and found novel factors towards efficient hematopoiesis. During mid-phase, application of basic fibroblast growth factor (bFGF, 10 ng/ml) under 1% O2 significantly increased numbers of CD43+ cells by 5-fold, as compared to cells without bFGF under 21% O2. Administration of a MYC inhibitor (50 μM) to cells during the early-phase, and tumor growth factor beta (TGF-beta) receptor inhibitor (SB431542, 10 μM) during mid-phase, also stimulated generation of CD34+CD43+ HPCs (1.5-fold and 3-fold in increase respectively). Notably, although this protocol resulted in a prominent yield of mesodermal progenitors during the early phase and of HPCs during the late phase (which were 50% and 10% of all derivatives from human iPSCs, respectively), this signaling manipulation had the opposite effect at other stages. For example, bFGF or TGFbeta receptor inhibition significantly depressed HPCs during the early-phase. Our co-culture system did not require factors known to affect *ex vivo* human CD34+ HSC / HPC expansion from cord blood cells, which contain stem cell factor, thrombopoietin, FMS-like tyrosine kinase 3 ligand, erythropoietin, interleukin (IL)-3 and IL-6. Comparing between with and without MYC inhibitor in early phase, MYC inhibitor gave rise to higher potential on the creation of hematopoietic cells in late-phase. Our novel culture protocol implicates new players in the stepwise development from a pluripotent state to blood cell generation. These players appear to be governed by circumstances resembling a developmental niche with lower oxygen conditions.

Poster Board Number: T-3072

EPIGENETIC REGULATION OF STEM CELL FATE: ELUCIDATING THE ROLE OF HISTONE MODIFICATIONS AND DNA METHYLATION DURING THE GENERATION OF PANCREATIC LINEAGES FROM HUMAN EMBRYONIC STEM CELLS

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Embryonic stem (ES) cells represent a potential novel source of insulin producing β-like cells for treatment of type 1 diabetes mellitus. When ES cells are differentiated, key genes controlling cellular fate are up- or down-regulated on the transcriptional level via different epigenetic mechanisms, including DNA methylation, miRNA silencing and chromatin remodelling by histone modifications. Understanding these mechanisms is pivotal for the development of novel differentiation protocols to generate β-cells from human ES cells. In this project we focus on the role of DNA methylation as well as histone methylation and acetylation in the differentiation of human ES cells towards pancreatic progenitors. This will be done by lentiviral-mediated knock down/overexpression studies using short hairpin RNA (shRNA) libraries against a broad spectrum of DNA methyltransferases, histone methyltransansferases/demethylases and histone acetyltransferases/deacetylases. Using a quantitative RT-PCR (qPCR) array, we observed that the expression of several epigenetic modifiers is changed during the generation of definitive endoderm from ES cells, indicating a differentiation induced change in the epigenetic landscape. The expression pattern and function of these enzymes will be mapped using methylated DNA immunoprecipitation (MeDIP), chromatin immunoprecipitation with sequencing (ChIP-Seq), qPCR and western blotting. During the knock down/overexpression studies, the cellular fate of the ES cells will be monitored using qPCR and quantitative immunocytochemistry (qICC). With this knowledge we hope to elucidate some of the epigenetic mechanisms regulating the cellular fate of ES cells, and use this to improve the strategies used to guide the differentiation of human ES cells towards pancreatic progenitors.

Poster Board Number: T-3073

OLIGODENDROCYTE AND MOTONEURON PROGENITORS DERIVED FROM HESC PROMOTE ASTROGLIOGENESIS FOLLOWING COMPLETE SPINAL CORD TRANSECTION THROUGH NOTCH AND JAK/STAT SIGNALING

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Spinal cord injury (SCI) results in neural loss and consequently motor and sensory deficit below the injury. Neural progenitors derived from hESC and iPS cells neural induces locomotor improvement following their transplantation into the animal models of SCI, but little is known about the effects and the underlying mechanism of these grafted cells on local tissue and endogenous neural stem cells. Recently, we have reported the regenerative effects and significant improvement of locomotor function in complete transection rat model of SCI following transplantation of oligodendrocyte progenitors cells (OPC) and motoneuron progenitors (MP) derived from hESC. In the present study, we further analyzed the underlying cell and tissue mechanisms of functional recovery after cell transplantation of OPC and MP hypothesized that beneficial effect is mediated by regenerative signalling pathways activated in the host tissue by transplanted cells. Here we show that transplantation of hESC-derived OPC and MP promote astrogliosis, thorough activation of jagged1-dependent Notch and Jak/STAT signalling supporting axonal survival.

Poster Board Number: T-3074

THREE-DIMENSIONAL SCAFFOLDING FOR LINEAGE SPECIFICATION OF HUMAN EMBRYONIC STEM CELLS

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Generation of pancreatic insulin-producing cells from human embryonic stem cells (hESCs) has arisen as one of the attractive cell-based therapies for diabetes treatment. Previously, we have demonstrated that a three-dimensional (3D) scaffold culture system can advance insulin secreting β-cell differentiation of mouse embryonic stem cells. In this study, we revealed that 3D scaffolding can significantly promote hESCs pancreatic lineages as well. A fine porous fibrillar network structure of collagen scaffold fabricated for pancreatic differentiation of hESCs was observed using SEM graphic analysis. The definitive endoderm (DE) differentiation is the first step and also the most critical step of hESC pancreatic differentiation. We observed a 5.5 fold increase in DE differentiation efficiency when hESCs were exposed to physiological cues provided by a 3D scaffold designed for hESC DE differentiation. Interestingly, blending distinct ECMs to the scaffold shows remarkable impact on hESC lineage specification. It was found that the expression of Sox17, Foxa2, and CXCR4, three DE marker genes increased 79

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folds when hESCs were differentiated in ECM-blended scaffolds. Immunofluoresence staining revealed a high level expression of DE marker proteins in these differentiated cells within 3D scaffolds. No significant cell apoptosis was observed under 3D environments. In particular, SEM imaging revealed abundant cell-cell and cell-matrix interactions in these cells within scaffolds. These results suggested that niches offered by a 3D scaffold play key roles in regulating pancreatic lineage specification of hESCs. Further efforts along this direction will lead to the development of an efficient hESC differentiation technology for producing mature β cells for cell-based diabetes therapy.

Poster Board Number: T-3075

ENHANCING THE DIFFERENTIATION EFFICIENCY OF HUMAN EMBRYONIC STEM CELLS TOWARDS CARDIOMYOCYTES BY ISL1 PROTEIN TRANSDUCTION

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Human embryonic stem cells (hESCs) have the potential to provide an unlimited source of cardiomyocytes that offers a precious tool for drug discovery and regenerative medicine. However, this application is limited due to insufficient efficiency of differentiation protocols. Several studies have shown that by over expression of cardiac transcription factors such as ISL1 the differentiation efficiency can be improved but genetically manipulation of hESCs is difficult and differentiated cells are not safe enough to be applied in cell therapy. To overcome these problems, we investigated the effect of direct introduction of ISL1 recombinant protein, as an alternative of genetic manipulation, in to the cells. Here we present an efficient protocol for differentiation of hESCs in suspension by direct introduction of a LIM homeodomain transcription factor, Islet1 (ISL1) recombinant protein into the cells. To this end, ISL1 ORF was cloned into the pENTR-D/TOPO Gate way entry vector. The ISL1 sequence from pENTER D-TOPO/ISL1 entry clone was transferred into the pDest17 Gateway and transformed to E-coli strain BL21 (DE3) by heat shock method. The sequence of ISL1 was verified by DNA sequencing. Recombinant fusion protein expression was then induced by the addition of Isopropyl-d-thiogalactopyranoside (IPTG). The expressed His6-TAT- ISL1 fusion proteins (rISL1) were purified by immobilized metal affinity chromatography (IMAC). The purified protein was analyzed by SDS-PAGE and Matrix-assisted laser desorption/ionization-tandem time of flight mass spectrometry (MALDI TOF/TOF MS). Interaction of the rISL1 protein with DNA was confirmed using gel shift assay, then the penetration of the protein into the hESCs was checked using immunostaining with both anti ISL1 and TAT antibodies. After protein expression and testing its functionality, the recombinant protein was applied for differentiation into cardiac cells. To find the best time of protein addition during differentiation, qRT-PCR was performed in every day of the first 15 days after differentiation induction. Differentiation was performed by addition of Activin A 100ng/ml for one day followed by 4 days treatment by BMP4 10ng/ml in RPMI medium containing 2% B27. Our data showed that ISL1 is expressed during day 2-8 after differentiation initiation. After that to find the optimum concentration of rISL1, cells were treated by different concentration of the protein (0, 10, 20, 30 and 40 µg/ml). We found the highest beating aggregates were derived by continuous treatment of hESCs by 40 µg/ml rISL1 protein during day 2-8 after differentiation initiation and by this method more than 70% of the colonies could beat. The treatment resulted in up to 3 folds increase in the number of beating areas; In addition, the number of cells which expressed cardiac specific markers (cTNT, CONEXIN 43, ACTININ, and GATA4) was doubled. This protocol was reproducible for another hESC line and caused increasing in the number of beating areas and expression of cardiac genes. In comparison with other current protocols for differentiation of hESCs into cardiomyocytes, our study presents a new efficient, safe, and reproducible procedure for cardiomyocytes differentiation. Our results will pave the way for scaled up and controlled differentiation of hESCs needed for biomedical applications in a bioreactor culture system.

Poster Board Number: T-3076

ESTABLISHMENT OF EPIGENETIC REGULATORY MECHANISM OF HEPATOCYTE DIFFERENTIATION USING HUMAN EMBRYONIC STEM CELLS.

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Human hepatocyte is essential tools for discovering new drugs and drug metabolism research. However, it is not easy to obtain fresh primary hepatocyte in a stable manner and culture for research use. There are a number of reports for hepatocyte differentiation using human stem/progenitor cells. Recently embryonic stem cells have been reported to efficiently differentiate into hepatocyte. However, there are few reports regarding the specific markers and their regulation applicable for purification of hepatocyte progenitors and their progenies. We focused on elucidating epigenetic regulatory mechanisms for markers highly expressed during the hepatocyte differentiation of human embryonic stem cells such as SOX17 and alpha-fetoprotein. In order to differentiate human embryonic stem cells into hepatocyte, we adopted a step-wise differentiation protocol consisted with initiation, differentiation and maturation. We analyzed histone acetylation and methylation profiles and DNA methylation status of SOX17 and alpha-fetoprotein at initiation(definitive endoderm) and hepatic differentiation(hepatocyte progenitor) steps. These results might be used for establishing regulatory networks for hepatocyte lineage marker expression and further utilized for discovering new hepatocyte(progenitor) markers.

Poster Board Number: T-3077

EFFICIENT DIFFERENTIATION OF HUMAN PLURIPOTENT STEM CELLS INTO PERICYTES

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In capillaries and other microvessels endothelial cells (ECs) are covered by a subtype of perivascular cells termed pericytes. Pericytes are critical regulators of vessel stability: disruption of normal EC-pericyte interaction in diseases such as hereditary hemorrhagic telangiectasia (HHT) leads to thinwalled dilated vessels which are the cause of recurrent bleedings. In addition pericytes share features of multipotent mesenchymal stem cells (MSCs) and can give rise to osteocytes, chondrocytes and adipocytes in vitro. Induced pluripotent stem cells (iPSCs) derived from patients with genetic diseases are promising tools to study mechanisms of disease and for drug and toxicology testing. We have derived multiple hu-

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man iPSC lines from control subjects as well as from HHT patients. Human iPSCs were generated by retroviral overexpression of the four Yamanka factors. Undifferentiated iPSCs on matrigel/mTESR medium expressed a set of typical markers of PSCs (OCT3/4, Nanog, TRA-1-81) and were able to differentiate into derivatives of the three germ layers in vitro. A prerequisite for the applications mentioned above is the successful derivation of the desired cell type, in particularly pericytes/vSMCs. We developed an efficient protocol for derivation of pericytes from hPSCs grown as colonies on matrigel/mTESR medium. To induce mesodermal differentiation embryoid bodies (EBs) were generated in low attachment plates in the presence of serum and VEGF and subsequently plated on gelatin in the presence of VEGF and the ALK4/ALK5/ALK7 inhibitor SB-431542. SB significantly increased the total yield of cells in the differentiating culture. Characterization of differentiating EBs at day 12 resulted in high percentages of CD146+ mesenchymal cells (>50% for multiple hPSC lines tested). Additional characterization revealed expression of known markers of MSCs (CD73, CD105) and perivascular cells (NG2, PDGFRβ). Pericytes were partially positive for smooth muscle actin but lacked expression of contractile smooth muscle cell proteins (smooth muscle myosin heavy chain and calponin). Culture of CD146+ cells on collagen IV-coated plates in MSC culture media (MEM-10FBS) resulted in the generation of a homogeneous pericyte population which could be expanded for 15-20 passages. Interestingly, supplementation with FBS and FGF2 significantly potentiated cell growth, while TGF-β inhibited cells proliferation. TGF-β mediated growth inhibition could be reversed by the addition of SB. Pericytes could be readily differentiated into osteocytes and adipocytes. Furthermore pericytes were able to stabilize in vitro networks of human umbilical vein endothelial cells (HUVECs). Assessment of the *in vivo* potential of PSC-derived pericytes is currently ongoing.

Poster Board Number: T-3078

HISTONE H3K27ME3 DEMETHYLASES MODULATE DEFINITIVE ENDODERM DIFFERENTIATION FROM HUMAN ES CELLS BY MODULATING THE WNT SIGNALING PATHWAY

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In human ESCs, poised genes, including those that encoding developmental transcriptional factors, exhibit bivalent mark with histone H3 lysine27 trimethylation (H3K27me3) and histone H3 lysine4 trimethylation (H3K4me3). Loss of H3K27me3 mark is believed to be crucial for the rapid expression of cell type-specific regulators during differentiation. However, how loss of this mark contributes to specific cell lineage differentiation is currently unknown. Here, we show that during definitive endoderm differentiation, the H3K27me3-specific demethylases KDM6A and KDM6B is increased with both are enriched in purified definitive endoderm cells. Knockdown of KDM6A/KDM6B does not affect human ESC maintenance but impaired their capacity of definitive endoderm differentiation. Further studies establish that KDM6A/KDM6B contributes to definitive endoderm differentiation through regulating the WNT signaling pathway. Knock-down of KDM6A/KDM6B results in decreased expression of WNT genes that impaired the respond to endoderm differentiation signals. Importantly, treatment with growth factors and chemicals regulating WNT signal can rescue the KDM6A/B knockdown effect of endoderm differentiation. Collectively, these results suggest that the H3K27me3 demethylases are required for robust endoderm differentiation from human ESCs, and this requirement involves the modulation of the WNT signaling pathway.

Poster Board Number: T-3079

THE USE OF HUMAN EMBRYONIC STEM CELLS TO ELUCIDATE THE ROLE OF CD4 IN MACROPHAGE DIFFERENTIATION AND FUNCTION

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We are utilizing a novel system we have recently developed involving the derivation of functional human monocytes/ macrophages from human embryonic stem cells (hESC) to closely examine the role of the CD4 molecule in monocyte/macrophage development and function. CD4 has an important role in the human immune system and in the pathogenesis of HIV. One of the primary targets and an important and poorly understood reservoir for HIV in an infected individual are monocytes/ macrophages. Other than allowing HIV infection of these cells, the role of the CD4 receptor in the function and development of monocyte/macrophages is not known. We have recently determined that ligation of CD4 on monocytes/ macrophages modulates gene and cytokine protein expression as well as macrophage differentiation. We are currently interested in further examining the roles and functions of the CD4 molecule on the monocyte/ macrophage cell subset. As a part of our analysis, we would like to examine the effects of the ablation of CD4 expression on macrophage biology. Interestingly, mouse macrophages do not express CD4 receptor at any stage of their differentiation, hence genetic ablation of this receptor in a murine model would not yield any information in this regard. Thus, we will utilize a hESC-based system that we can first genetically manipulate and then examine monocyte/ macrophage differentiation and function from a cell type that is among the earliest in human hematopoietic development. To accomplish this goal, we constructed lentiviral vectors expressing a GFP reporter gene and either a siRNA specific for CD4 molecule, or a control siRNA with a scrambled sequence. After initial testing for the ability to downregulate CD4 expression on cell lines and primary macrophages, we introduced these vectors into H1 hESC. The transduced GFP+ cells were sorted out and propagated on MEFs for more than ten passages, establishing the lack of toxicity of transgenic siRNAs in hESC. Next, we differentiated these transgenic hESC into macrophages using a two-step procedure. As expected, we observed reduced expression of CD4 receptor on the resulting macrophages, suggesting that this protein may not play a major role in macrophage development, at least in an *in vitro* system. We are currently investigating if CD4 knockdown influences the function of these transgenic macrophages and if these cells are susceptible to HIV infection. In summary, it is anticipated that these studies will shed light on the potential roles and importance of CD4 in monocyte/macrophage development and/or function. Further, the use of embryonic stem cells in this research, due to their high plasticity, self-renewal ability, and ability to be genetically manipulated, may allow the development of immune replacement therapeutic strategies to be developed for HIV and other similar diseases.

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Poster Board Number: T-3080

IDENTIFICATION OF SMALL MOLECULE KINASE INHIBITORS CAPABLE OF MODULATING THE MESENDODERM DIFFERENTIATION PATHWAY IN HUMAN ES CELLS.

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Human pluripotent stem cells provide a powerful platform for modeling human diseases and development. Herein, we describe a screen of kinase inhibitors aimed at identifying pathways involved in early germ layer specification. Starting from a master cryopreserved stock of H9 human embryonic stem cells, we first established conditions capable of reproducibly inducing 40-50% mesendoderm formation as judged by the nuclear co-localization of SOX17 and EOMES protein. Once the baseline differentiation conditions were established, we performed a primary screen of 301 compounds with known kinase inhibition profiles. The kinase inhibitor library was assembled to provide broad coverage of the known kinome. Approximately 100 compounds in the library were considered "highly selective" (inhibiting 1-10 kinases), 100 compounds "moderately selective" (inhibiting 10-25 kinases), and 100 "promiscuous" (inhibiting >25 kinases). During the primary screen, individual wells of differentiating H9 cells were treated on day 1 and day 2 with 5uM of test compound or a DMSO vehicle control. Following 3 days of differentiation, the cells were fixed, stained with antibodies against SOX17 or EOMES and analyzed using immunofluorescent-based high-content imaging. 60 compounds were found to be cytotoxic, 20 compounds reduced both SOX17 and EOMES expression without overt cytotoxicity, 14 compounds increased both SOX17 and EOMES expression, and 2 compounds increased EOMES without a concomitant increase in SOX17 expression. Current efforts have focused on the compounds that increased expression of both SOX17 and EOMES. The 14 compounds identified in the primary screen were subject to secondary screening consisting dose response treatment (10uM, 5 uM and 1uM) with each compound. From this group, 5 compounds were found to reproducibly induce SOX17 in the secondary screen. Two of the compounds increased the endoderm differentiation significantly (~95% of cell population are Sox17 positive at Day 3), and targeted the same kinase. Experiments are underway to examine the signaling pathways modulated by the compounds, examine their effects in additional independent human ES and iPSC cell lines, and to determine if any combination of small molecules can efficiently drive mesendoderm differentiation in the absence of other protein growth factors. If successful, these compounds may help to not only increase the efficiency of differentiation to specific cell types, but also to provide a first tool to mechanistically separate endoderm and mesoderm differentiation pathways.

Poster Board Number: T-3081

THE ROLE OF MHC CLASS I IN THE IMMUNOGENIC PROPERTIES OF HUMAN PLURIPOTENT STEM CELLS AND THEIR DIFFERENTIATED DERIVATIVES

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Human pluripotent stem cells (hPSCs) are a promising source for cell replacement therapy because they can replicate indefinitely and generate any cell type in the body. However, in order to use these cells clinically we need to determine whether it will be necessary to immune match them to the recipient. The Major Histocompatibility Complex (MHC) plays an important role in graft rejection by allowing immune cells to distinguish "self" from "non-self." Recent studies have shown hPSCs have low expression of MHC proteins and may be "immune privileged." However, it is not known whether differentiated cell types derived from hPSCs retain low expression of MHC or how germ layer-specific cell types will respond to human immune cells. We have demonstrated by gene expression analysis that hPSCs show increased expression of MHC class I upon differentiation in vitro into cells from each of the three embryonic germ layers including neurons, cardiomyocytes and hepatocytes. In addition, microarray data collected from fetal and adult tissue show a similar trend, with MHC class I expression gradually increasing during *in vivo* development. As part of our ethnic diversity iPSC project, we have derived iPSCs from individuals with different MHC haplotypes and are using them to investigate in vitro immunogenicity of hPSCS and their germ layer-specific differentiated derivatives at various stages in their development. In our first studies we are using two iPSC lines with very different haplotypes: a Yoruba haplotype (HLA A*23:03/ A*30:02; HLA B*57:03/57:03; HLA C*18:01/18:01) and a Caucasian haplotype (HLA A*01:01/ A*02:01; HLA B*08:01/37:01; HLA C*06:02/07:01). Data collected in these studies will be used to answer critical questions regarding the immune stimulating capabilities of cell types derived from hPSCs for future transplant studies.

Poster Board Number: T-3082

INDUCTION OF HUMAN ES CELL-DERIVED HEPATIC CELLS BY NOVEL METHOD USING LOW MOLECULE

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Human embryonic stem cells (hES cells) are expected to be applied for regenerative therapy, because they have ability of infinite proliferation and pluripotency. Surely many methods have been proposed to differentiate hepatic cells from hES cells, but most of them have poor efficiency for differentiation, expensive protocols using growth factors and are far from safety in the case of cell processing using virus transfection To solve these problems, we tried in this paper to establish the new original method using low molecule and to differentiate hepatocytes directly from hES cell without embryoid formation. The phenotypic changes of hES cell-derived hepatic cells were analyzed with RT-PCR, immnunostaing and real time RT-PCR in addition to morphologic and functional analyses. These results showed superiority of this method in simplicity, efficiency and economy, which guarantees to create one possible source of hepatic cell for cell transplantation.

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SPECIFIC MIRNAS EXPRESSION IN ERYTHROPOIETIC DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS

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Objective: microRNAs (miRNAs) are small non-coding RNAs that regulate gene expression at the post-transcriptional level through mRNA degradation or translation inhibition. However, it is not known whether miRNAs participate in hematopoietic or in particular erythropoietic processes. The aim of this study is to investigate erythroid lineage-specific miRNAs expression regulated during *in vitro* erythropoiesis using Human embryonic stem cells (hESCs) and human umbilical cord blood CD34+ cells. Materials and methods: CD34+ hematopoietic stem/progenitor cells were produced from hESCs *in vitro* and subsequently induced to differentiate into erythroid cells by culture on OP9 feeder cells followed by erythropoietic differentiation culture using specialized conditioned culture media. The expression profiles of four lineage-specific miRNAs, miR-142-3p, miR-142-5p, miR-146a and miR-451 during erythropoiesis were analyzed by quantitative PCR of extracted miRNAs. Results: Expression levels of these miRNAs were dynamically changed during differentiation of hESCs to CD34+ hematopoietic stem/progenitor cells and subsequent differentiation of CD34+ cells into erythrocytes. Conclusion: This result indicates that miR-142-3p, miR-142-5p, miR-146a and miR-451 express specifically during erythropoietic induction of hESCs. This suggests that these four miRNAs might involve in regulating erythropoiesis.

Poster Board Number: T-3085

DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS INTO MEDIUM SPINY NEURONS OF THE STRIATUM

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The objective of this work is to develop a high purity human stem cell based platform for Huntington's Disease (HD) mechanistic research and drug discovery. HD is a neurodegenerative genetic disorder that results primarily in the loss of medium spiny projection neurons (MSN) of the striatum. Current animal models do not sufficiently recapitulate the complex cascade of neurodegenerative events in the human. Current cellular models are complicated by primary extraction and purification methods or are confounded by contaminant progenitor populations. The need exists for high purity MSN and lateral ganglionic eminence progenitors (LGP) at multiple developmental stages for transplant studies as well as drug discovery and predictive toxicology assays. Genetically abnormal blastocysts donated by participants were thawed and an hESC line was derived and maintained in animal-free conditions and characterized. hUCI-HD1 is a karyotypically normal HD line carrying 44 CAG repeats. In addition, a genetically normal hESC line and an iPSC line carrying 180 CAG repeats were differentiated side-by-side. Undifferentiated hESC and iPSC were grown on matrigel-coated flasks to subconfluence in a feeder-free system. Cultures were then transitioned from conditioned medium to a DMEM-F12 rich medium for neural induction. Cells were grown in suspension and passaged routinely in differentiation medium supplemented with growth factors until day 60, when they were replated and growth factors were withheld for final maturation to MSN by day 66. Stage-specific immunocytochemistry was performed at day 14 (GSH-2), day 25, day 42 and 45 (FoxP1) and day 66 (DARPP-32) to optimize growth conditions. Cultures were additionally matured to day 135 and electrophysiology was performed, demonstrating neuronal firing patterns and functional synaptic connections. During recording of spontaneous post-synaptic currents, NBQX/APV and GABAzine were used to block glutamatergic and GABAergic events, showing that the cells received both excitatory and inhibitory inputs. The early stage LGP will be used for studies of striatal development and the MSN will be used for transplant studies, drug discovery and predictive toxicology.

Poster Board Number: T-3086

POSSIBLE USE OF HUMAN PLURIPOTENT STEM CELL-DERIVED CARDIOMYOCYTES AS AGING MODEL FOR HUMAN CARDIAC CELLS

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Human embryonic stem cells (hESCs) have abilities to self-renew and differentiate into all types of cells in human body including cardiac cells. These features of hESCs make them considered as an ideal model for developmental biology, human development and drug screening. Aging of cardiomyocytes (CMs) is natural phenomenon in human cardiac cell, however, aging could not be studied using human cells. In this study, we analyzed aging phenomenon in hESC-derived cardiac cells and investigate the possibility of using hESC-derived CMs as aging model. We induced differentiation into CMs from hESCs using direct differentiation and we classified hESC-derived CMs into early (day 12), middle (day 18) and late (day 24)-stage, based on their functionality. We observed morphology of hESC-derived CMs and counted beating rate. In addition, we performed senescence-associated (SA) β-gal staining and counted positively-stained hESC-derived CMs. We performed immunostaining in each stage of hESC-derived CMs and confirmed the cardiac features of differentiated CMs. Middle and late-staged hESC-derived CMs showed increase of pigmented cells and demonstrated slower beating late due to in vitro culture period. Human ESC-derived CMs at day 12 showed small population of stained cells. And, CMs at day 18 and -24 showed larger population of stained cells and the positively-stained cells were abundant in day 24 hESC-derived CMs. In this study, we demonstrated the *in vitro* stage-dependent aging phenomenon in hESC-derived CMs. Furthermore, we insist the possible usage of hESC-derived CMs for aging study of human cells (2011-0007944 and A111539).

Poster Board Number: T-3087

MAINTENANCE OF THE FUNCTIONAL RETINAL PIGMENT EPITHELIAL CELLS DERIVED FROM HUMAN EMBRYONIC STEM CELLS

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Maintenance of the functional retinal pigment epithelial (RPE) cells is important issue for cell replacement therapy of retinal degenerative disease because it is related to functional recovery. We regenerated functional RPEs as well as mature neurons from

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human embryonic stem cells (hESCs) derived spherical neural masses (SNMs). SNMs are pure cell masses of hESC-derived neural precursors and they are differentiated into neurons by attachment culture. However, cystic portions of some SNM can be differentiated into RPE cells have functional characteristics such as pigmentation and expression of mature marker. RPE cells derived from cystic portions of the SNMs also can be expanded by monolayer culture. However, during expansion, functional characteristics are reduced rapidly. For maintenance of the functional characteristics or re-maturation of the expanded RPEs, we test some co-culture systems. In some cases, RPE pigmentation was maintained and expression of the mature marker was also detected. RPEs can be purified from co-culture system easily.These strategies in our study could be helpful for functional recovery after RPE transplantation. This research was supported by Mid-career Researcher Program through NRF grant funded by the MEST, Korea (No. 2009-0084597).

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EXAMINATION OF CELL SIGNALING PATHWAYS DURING EARLY ENDODERM SPECIFICATION FROM HUMAN EMBRYONIC STEM CELLS UTILIZING FLUORESCENCE CELL BARCODING AND INTRACELLULAR FLOW CYTOMETRY

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The ability of human embryonic stem cells (hESCs) to differentiate into various cell lineages allows for the study of developmental biology and has applications in the fields of regenerative medicine and cellular therapy. To optimize differentiation protocols and to answer biological questions, it is important to understand the signaling pathways that are involved in lineage specification. We applied a multiparametric flow cytometric screening approach to analyze cell signaling during endoderm differentiation from hESCs. H9 hESCs were differentiated to early endoderm in the presence of low serum and Activin A. Our differentiation method was verified using multicolor flow cytometry to monitor the downregulation of the pluripotency marker Nanog and the upregulation of endoderm markers Sox17 and FoxA2. We screened cells at different time points of endoderm differentiation by flow cytometry using over 80 antibodies specific to proteins of many known signaling pathways. We utilized fluorescent cell barcoding to increase throughput and minimize experimental variability, which enabled multiple differentiation time-points to be analyzed in a single sample. Data from the screen suggest a complex signal transduction network for endoderm specification of pluripotent stem cells in culture. We observed changes in the phosphorylation states of Rb and S6, which are implicated in cell proliferation. In addition, we detected changes in multiple proteins involved in SMAD and integrin signaling pathways, which have been implicated in self-renewal and differentiation. We also observed changes in markers involved in B-cell signaling, suggesting this signaling pathway may play an additional role during stem cell differentiation. Further analysis of our data set may reveal additional signaling pathways that are relevant to endoderm specification. This screening methodology can be readily applied to various stem cell populations and their derivatives to explore cell signaling events such as self-renewal, reprogramming, and lineage specification.

Poster Board Number: T-3090

NEW BMP INHIBITOR DORSOMORPHIN ROBUSTLY ENHANCES THE DIFFERENTIATION EFFICIENCY OF HUMAN EMBRYONIC STEM CELLS INTO RETINAL PIGMENT EPITHELIUM

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Functional retinal pigment epithelium (RPE) can be generated from human embryonic stem cells (hESC). These cells hold great promise as a potentially unlimited source of material for the treatment of retinal disorders such as age-related macular degeneration. Current differentiation methods rely on spontaneous differentiation or the use of expensive recombinant growth factors such as noggin. Dorsomorphin (DM) is a highly potent small molecule that targets several TGFβ type receptors and interferes with both BMP and activin/nodal type signalling, resulting in high efficiency neural conversion. Being a small molecule it is less expensive, more stable and better able to penetrate large cell masses than the proteinaceous equivalent. In this study we have investigated the ability of early DM treatment to direct hESC-RPE differentiation. Cells from the hESC line Shef6 and Shef3 were differentiated in a monolayer on feeders or feeder-free in the presence of 1μM DM. Pax6 expression was measured after 15 days to determine neural conversion in DM treated vs. control wells. After a 50 day differentiation protocol, the RPE yield was assessed by quantitative PCR and by comparing the increase in surface pigmentation over time. A significant increase in pax6 expression in the presence of DM was detected at day 15 by QPCR and immunocytochemistry. This was coupled with a five fold average increase in pigmented surface area relative to untreated controls. Intriguingly the presence of mitotically inactivated mef feeders or feeder conditioned media appears to act synergistically with DM to enhance both neural conversion and RPE differentiation in both Shef3 and Shef6, a phenomenon that requires further investigation. Efficient and simple protocols for generating large quantities of RPE will be important for progression in research and development and for clinical use. RPE can be reliably derived from a number of HESC and IPSC lines but efficiency varies greatly, with some lines unable to differentiate into RPE at all. In this protocol we demonstrate enhanced RPE yields in feeder-free conditions without the need to make embryoid bodies or use expensive amounts of recombinant proteins. Dorsomorphin provides a simple, cost-effective, one-step method for enhancing neuroectodermal differentiation and enhancing hESC-RPE differentiation efficiency.

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IDENTIFICATION AND PROSPECTIVE ISOLATION OF SOMITE-LIKE CELLS FROM MURINE AND HUMAN PLURIPOTENT STEM CELLS

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Due to their intrinsic developmental potential and proliferative ability, human embryonic stem cells (hESCs) hold remarkable promise for their use in cell-based therapies as well as an in vitro model for early human development. Although significant advancements in the derivation of cells with specific neural (CNS, neural crest), endoderm (liver, pancreas) and lateral mesoderm (blood, endothelium) fates have been made, little has been done on the refinement of current techniques for the differentiation of hESCs into derivatives of paraxial mesoderm, such as skeletal muscle. We have set up cul-

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ture conditions to specifically induce hESCs to differentiate towards a combination of neural and not neural fates in a monolayer culture system. After 30-35 days of differentiation we have observed the appearance of a distinct cell population growing in tight clusters displaying skeletal muscle differentiation ability. Due to its unique cell morphology, this cell population could be easily mechanically isolated for further analyses and characterization. Immunostaining and RT-PCR analyses performed on these cells revealed a pattern of expression similar to the one of the somites, the embryonic structures from which the skeletal muscles of the trunk and limbs originate. Somite molecular markers such as Paraxis, Meox2, Eya2, Six1, Pax1 and Pax3 were among the ones expressed. Most significant was the expression of Pax3, a molecular marker initially widely expressed in the somites and whose expression is later confined to the dermomyotome, the portion of the somites that gives rise to muscles. Based on these findings, we speculated that this novel cell population, named Somite-Like Progenitor (SLP) cells, could represent an in vitro equivalent of the earliest muscle progenitors emerging in the embryo. Confirmation was attained through the SLP cells' ability to differentiate into myogenin+ mature cells in vitro under defined conditions. Significantly, we were also able to isolate and subsequently re-expand SLP cells utilizing selective FACS sorting strategies based on a unique combination of surface markers that included CD29. Furthermore, we have observed the appearance of cell clusters with the same unique morphology of SLP cells in differentiating mouse ESC (mESCs). The mESC-derived cell population also exhibited skeletal muscle potential in vitro and had an almost identical gene expression profile as the hESC-derived SLPs. Isolation of mESC-derived SLP will help setting up *in vivo* strategies to unveil the full potential of these cells. The developmental progression from undifferentiated ESC toward the specification of muscle cells is largely unexplored in humans. The generation of hESC derived somite-like cells, not only will give new insights into human skeletal muscle development, but will also accelerate the move towards the use of hESC-derived skeletal muscle cells for therapeutic use in a variety of muscle atrophy and wasting conditions.

Poster Board Number: T-3092

IDENTIFICATION OF SURFACE MARKERS ON HUMAN PLURIPOTENT STEM CELLS AND THEIR DIFFERENTIATED PROGENY

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The self-renewal and differentiation potential of human pluripotent stem (hPS) cells offers a virtually unlimited source of therapeutic replacement cells to treat a variety of degenerative diseases including, arthritis, osteoporosis, heart disease, Parkinson's, Alzheimer's and macular degeneration. The ability to reprogram adult cells to induced pluripotent stem (iPS) cells has enabled the creation of patient-specific hPS cells as a source of cells for disease modeling, drug discovery, and eventually cell replacement therapies. While, reprogramming technology has dramatically increased the availability of normal and diseased hPS cells for basic research, a major bottleneck is the critical unmet need for more efficient methods of deriving well-defined therapeutic replacement cell populations free of residual hPS cells. A current limitation is the lack of surface markers both on hPS cells and various progenitor cell types. Phage display is a powerful method for identifying affinity reagents against cell surface target molecules. However, identification of specific progenitor cell-binding peptides by selection of a phage display library against differentiating hPS cells is difficult because cellular heterogeneity limits the availability of any one particular progenitor target cell type. To circumvent this problem, we selected phage display libraries against clonally pure human embryonic progenitor (hEP) cell lines from the ACTcellerate™ cell line collection, which consists of over 140 scalable lines. A key advantage of this strategy is that it enables peptide selection against large numbers of a single clonally pure cell type which might otherwise be represented by only a few cells in a mixed population of differentiating hPS cells. Using this approach, we have successfully identified cell-targeting peptides with high specificity and selectivity for progenitors of cartilage and bone. Specificity of the targeting peptides was demonstrated in peptide phage competition experiments with excess free peptide. Selectivity was demonstrated using immunocytochemistry to assess peptide phage binding against the target cell line compared to other hEP lines and undifferentiated hPS cells. We are currently selecting peptides against additional progenitor cell types (i.e. adipose, smooth muscle) and testing the use of labeled peptides in FACS- based cell separations to enrich mixed cell populations for specific progenitor cell types such as cartilage and bone. In an effort to develop methods of removing residual pluripotent stem cells, we have identified a novel surface antigen, termed C7, on hPS cells against which we are preparing antibody and peptide affinity reagents. In a model experiment, osteogenic progenitor cells were mixed 1:1 with hPS cells and enriched to >99% purity in a single round of hPS removal by C7-based FACS. Our data suggest that phage display can be used to identify progenitor and hPS cell targeting peptides that will be useful for the production of well-defined cell populations free of potentially tumor forming hPS cells.

Poster Board Number: T-3093

BIOPROCESS STRATEGIES FOR MINIMISING HETEROGENEITY DURING EXPANSION OF MURINE EMBRYONIC STEM CELLS (MESCS)

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Pluripotent embryonic stem cells (ESCs) represent a potentially unlimited supply of cells for therapeutic use, developmental studies and toxicity testing. Since their discovery, our understanding of the nature of embryonic stem cell cultures has progressed. Research groups have now identified subpopulations within the pluripotent population that preferentially differentiate into specific lineages. Many differentiation protocols are currently long and inefficient, often resulting in low purity of the target cell type. This may be due, in part, to heterogeneous starting populations. For successful translation of ESC-derived cell therapies to the clinic it is necessary to develop robust and highly efficient differentiation processes. We hypothesised that purer end populations could be achieved with homogenous starting populations. We investigated two approaches to achieve this. Firstly, we investigated heterogeneity at the cellular level by establishing 40 single cell-derived clonal lines from wild-type mixed populations. Embryoid body differentiation highlighted clonal line developmental bias through comparative analysis of germ layer markers. These data support the notion that a specific clonal line could be chosen for a specific differentiation. Our second approach was to control microenvironmental factors at the expansion stage. We chose to investigate oxygen tension. Mouse early embryo development occurs under hypoxic conditions which suggests that traditional culture conditions may be sub-optimal. Firstly the effects of hypoxia on self-renewal and pluripotency were characterised. We then investigated the hypothesis

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that maintenance in hypoxic conditions has an effect on differentiation in traditional normoxic conditions. Our studies suggest two separate approaches for minimising the innate heterogeneity within pluripotent ESC cultures that may improve differentiation efficiency.

Poster Board Number: T-3094

ADAMTS-9 IS INVOLVED IN MESODERM DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS

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A Disintegrin-like And Metalloproteinase with Thrombospondin (TSP)-Type I sequence motifs 9 (ADAMTS9) is widely expressed in mouse and human fetal tissues. ADAMTS9 null mice cannot survive beyond E7.5 and its haploinsufficiency is associated with cardiac and aortic anomalies. We hypothesized that ADAMTS-9 was important during early embryogenesis. Human embryonic stem cell (hESC) was used as a model for studying ADAMTS9 expression and its role during early differentiation. Our results indicated that ADAMTS9 immunoreactivity was detected in cells located at the boundary of hESC colonies in undifferentiated state. Its mRNA and protein expression increased time-dependently in the first 24 days' of embryoid body (EB) formation. The expression pattern was similar to that of mesoderm and endoderm markers. The positive correlation of ADAMTS9 with ESC differentiation was also found in the mouse system, in which Adamts9 expression was increased time-dependently during mouse EB formation and down-regulated during reprogramming from somatic cells into induced pluripotent cells. During hESC differentiation ADAMTS9 was co-localized with several specific mesoderm and endoderm markers. Transient ADAMTS9 knockdown by siRNA in hESC significantly decreased the expression level of mesoderm marker, REN. Induction of differentiation of hESCs towards the mesoderm lineages dramatically increased ADAMTS9 expression of the differentiated colonies. In the differentiated mesodermal cells, ADAMTS9 was co-expressed with vascular endothelial markers, VEGF and CD31, but not with pericyte markers, alpha muscle actin. Lentiviral vector encoding ADAMTS9 shRNA was used for long term knockdown of ADAMTS9. Knockdown of ADAMTS9 significantly reduced the expression of certain mesoderm markers, REN, PDGFRα and CD34. In conclusion, ADAMTS9 was induced during mesoderm differentiation and its knockdown led to down-regulation of mesoderm markers. The roles of ADAMTS9 during hESC differentiation and early embryo development warrant further investigation. Acknowledgement: Seed Funding Scheme to Support Research Projects on Human Embryonic Stem Cells (ESC) and Induced Pluripotent Stem Cells (iPSC), Stem Cell & Regenerative Medicine Consortium (SCRMC), Li Ka Shing Faculty of Medicine, The University of Hong Kong

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BMP10, A HEART-SPECIFIC CYTOKINE, INDUCES DIFFERENTIATION OF HUMAN PLURIPOTENT STEM CELLS WITH A MUCH HIGHER POTENCY THAN MEMBERS OF THE BMP7 SUBGROUP

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Bone morphogenetic proteins (BMPs) are a large family of biologically active cytokines. BMP ligands are present within a broad range of animals where they are highly conserved. In humans they are known to have pleiotropic functions during all stages of life. With respect to human pluripotent stem cells, it has been shown that BMP2, 4, 6 and 7, either as homodimers or heterodimers, can induce differentiation into embryonic (mesoderm, endoderm) and extraembryonic (trophoblast) lineages. In differentiation protocols applying more stringent cell culture conditions, these BMPs have also been successfully used to differentiate human pluripotent stem cells into vascular progenitor cells, chondrocytes and primordial germ cells. However, these ligands only represent a minority of the BMP family, which consists of more than 20 members and can be divided into several subgroups, depending on sequential and structural homology. In addition, it has been shown for certain human adult stem cells and progenitor cells, that one and the same cell type can respond differently to distinct ligands, even if they belong to the same subgroup (e.g. BMP5, 6 and 7). This prompted us to investigate the effect of distinct members of the BMP family - that have to our best knowledge not been tested yet - in terms of their effect on human pluripotent stem cells. For this purpose, we chose BMP5, BMP13 (GDF6) and BMP10, all representing members of distinct subgroups, with BMP5 belonging to the BMP7 subgroup, as well as the well-researched BMP7 as a reference. Furthermore, we successfully reprogrammed human chorionic villi cells to induced pluripotent stem cells (hiPSCs). In our in vitro experiments we treated these hiPSCs - as well as human embryonic stem cells (hESCs lines H1 and H9) as a reference, with equal concentrations of these human recombinant BMPs in chemically defined medium (without addition of any other exogenous cytokines or inhibition of autocrine signaling pathways). We observed that hESCs as well as hiPSCs respond identically to all the four BMPs tested, namely they first differentiated to mesendoderm cells and, predominantly to early trophoblast cells (indicated by the expression of primitive streak markers T and MIXL1 and early trophoblast markers CDX2 and HAND1), followed by cells belonging to the definite mesoderm/endoderm and trophoblast lineage (indicated by cell fusion markers and as latest, secretion of hCG as marker of multinuclear syncytiotrophoblast cells). However, as we found out on mRNA (microarray, qPCR) and protein level (Western Blot, immunocytochemistry, ELISA), the kinetics and potency of these ligands differ immensely. Interestingly, we discovered that BMP10, whose expression *in vivo* is restricted to specific parts of the heart for a short time frame during heart development, induces differentiation with by far the highest kinetics/potency, and BMP13 being the weakest. Detailed comparative kinetic studies of earliest treatment responses - when differentiation of the cells had not occurred, revealed a significantly higher level of phosphorylated SMAD1/5 for BMP10 at any time point investigated, whereby signaling via non-SMAD-pathways (such as MAPKs and Akt) appears to play only a minor role. In summary, our studies have unveiled additional cytokines that could be applicable for lineage specific differentiation of hiPSCs/hESCs to derive donor cell types useful for cellular regenerative therapies in the future.

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AN IMMOBILIZED CHIMERIC FORM OF CELL ADHESION MOLECULE STIMULATES NEURAL CONVERSION OF MOUSE EMBRYONIC AND INDUCED PLURIPOTENT STEM CELLS

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The unique property of ES/iPS cells represents a promising source to overcome many diseases by providing an unlimited supply of

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many cell types, including neurons. The formation of appropriate neural circuits depends on a complex interplay between extracellular guiding cues and intracellular signaling. N-cadherin is one of the best-studied cell adhesion molecules (CAMs) involved in the formation of axon and dendrite (collectively termed neurite). However, exactly how N-cadherin influence the differentiation of ES/iPS cells into neural or glial progenitors remains to be clarified. To elucidate the role of this CAM, we introduced immobilized chimeric form of N-cadherin (N-cad-Fc) using two differentiation induction protocols: serum-free neurosphere (SFN) and hanging-drop-based embryoid-body (HDEB) strategy. In SFN and HDEB methods, the differentiated ES/iPS cells responded to immobilized N-cad-Fc chimera by extending longer neurites than controls. The differentiated cells on N-cad-Fc also showed almost similar effect in absence of any external neural stimulatory factors. The response was significantly reduced by pre-treating differentiated cells with neutralizing N-cadherin antibody or inhibitors to the fibroblast growth factor (FGF). Time lapse imaging also showed that N-cadherin substratum is capable of directing a neurite outgrowth response. In addition, the differentiation induction in adherent monoculture protocol using N-cadherin substratum showed its potentiality to remove undifferentiated or poorly differentiated populations from differentiated neural cells. The neural and glial subtypes were confirmed under transcript and protein level using regional specific differentiation markers. These data showed that the applicability of substrate-associated adhesion molecules to stimulate healthy neurons from ES and iPS cells and point to the potential of using cadherin-based chimeras to promote nerve regeneration.

Poster Board Number: T-3098

ISOLATION AND CHARACTERIZATION OF A2B5-POSITIVE CELLS FROM HUMAN EMBRYONIC STEM CELLS

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Currently, oligodendrocyte differentiation from human embryonic stem cells (hESCs) takes a long time. Therefore, we have developed a protocol for producing highly purified cells that exhibit OPC characteristics from hESCs in a short period of time. Embryonic bodies derived from hESCs were differentiated into neural precursor cells (NPCs) through simultaneous inhibition of BMP and Activin/Nodal signals with small molecules. NPCs expanded in N2B27 medium supplemented with bFGF and EGF for one week included lots of A2B5 immunoreactive cells which can subsequently give rise to cells with OPC characteristics. To get enriched A2B5-positive populations and to exclude unwanted cell population, we took advantage of the magnetic activated cell sorting (MACS) technique with A2B5 specific antibody. We confirmed that it is possible to get highly pure population of A2B5-positive cells (98%) after MACS by Fluorescence-activated cell sorting (FACS) and cell counting. After MACS, sorted A2B5-positive cells were expandedin N2B27 media supplemented with PDGF-AA, IGF-1 and bFGF for 10 days to consequently generate OPC-like cells expressing PDGF-Rand NG2 (70~80%). Majority of these cells had bipolar or tripolar morphology and expressed the transcripts of *PDGFR, NG2, Olig1/2, Sox families 8/9/10* which are associated with OPC differentiation. Interestingly, we found that the treatment of neuregulin (NRG) facilitated the proliferation and survival of PDGFR/NG2-positive cells and up-regulated genes of OPCs (*NG2, Sox10, Olig1/2*). As a result, 70~80% of total cells were OPC-like cells (A2B5+/NG2+/PDGF-R+) after ~4 weeks since the beginning of differentiation. When we transplanted hESC-derived OPCs into traumatically injured spinal cord model, OPCs transplanted group improved hindlimb performance in both legs after 3 weeks after transplantation. We observed strong MBP-immunoreativity in the cell-injected site of injured spinal cord and transplanted group significantly increased the amount of myelinated axons compared to control group. These findings suggest that OPCs could be fast and efficiently generated from hESCs by A2B5-mediated cell sorting and have potential benefits for diseases such as spinal cord injury.This research was supported by the Ministry of Education, Science, and Technology (MEST) (SC1110); National Research Foundation, MEST (2010-0020353), the Stem Cell Research Center of the 21th Century Frontier Research Program funded by the Ministry of Education, Science and Technology, Republic of Korea and from Korean Health Technology R&D Project, Ministry for Health, Welfare & Family Affairs (A100694)

Poster Board Number: T-3099

ANALYSIS OF EARLY REGULATORY FACTORS DURING NEURAL CREST CELL DIFFERENTIATION FROM MOUSE EMBRYONIC STEM CELLS.

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The neural crest (NC) is a transient multipotent stem cell population. They arise at the progenitor domain, neural plate border (NB) between the neural and non-neural ectoderm. They migrate through the embryo and give rise to a vast range of cells. Abnormal development or loss of the NC cells causes neurocristopathy. Studying the mechanism of NC differentiation allows us to understand the pathogenic mechanisms and to develop effective therapies of neurocristopathy. Previous studies using model animals and cultured cells showed that several transcription factors such as Slug, Twist, play important roles in NC differentiation. However, the regulatory mechanisms during the early step of NC differentiation are not fully understood. This is partly because the experimental models and methods still remain undeveloped to analyze the early NC differentiation accurately. We previously established the NC cell induction system from mouse ES cells in serum-free monolayer culture. This induction system is able to differentiate the mES cells into NC cells which express typical NC marker genes (e.g., *slug, AP2a*, *p75*, *c-kit*, *Twist*, *Sox9*) and differentiate into NC derivatives (e.g., peripheral neurons, oligodendrocytes, smooth muscle cells, and chondrocytes). This induction system enabled us to follow up the process of cell status change during NC cell differentiation. In this study, we attempted to identify the regulatory factors in early NC differentiation by the use of this induction system. For this purpose, we performed global gene expression analysis in mES cells just after the onset of NC differentiation. As results, we identified 53 genes whose expression increased from the onset of NC differentiation. Among these candidates, early transcriptional regulatory genes including *Msx*, *Dlx*, *Id* families, *Ap2a*, and *Snail*, which were involved in NC differentiation and NB specification were included. On the other hand, cytoskeletal and extracellular structure genes

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associated with NC differentiation were not included, suggesting that these candidates are mainly involved in the onset of NC differentiation. These candidates also include novel factors. We next confirmed *in vivo* expression of these factors. We further examined the role of the factors in early NC differentiation. The inhibition of one of the factor with antisense oligonucleotides decreased the expression of NC marker genes and reduced cell migration activity *in vitro*. In summary, we screened NC regulatory factors by using our established induction system which recapitulated the early step of NC differentiation *in vitro*. We identified a novel regulatory factor from the screen and analyzed the cell status change caused by the factors during NC cell differentiation. This system is particularly useful for studying of early NC development because we identified regulatory factors acting at the first step of differentiation by using this system. Our methods and findings demonstrated an effective strategy to understand the NC differentiation mechanisms.

Poster Board Number: T-3100

THE ROLE OF LHX2 IN HUMAN NEURAL FATE DETERMINATION

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The mechanistic basis of early neural development in human is still largely unknown. Human embryonic stem cells (hESCs) are pluripotent stem cells capable of giving rise to all the cell types of body thus provide us an excellent opportunity to explore the genetic and epigenetic mechanisms underscoring early neural development. LIM homeobox 2 (LHX2) is a transcription factor that was found highly expressed in the hESC-derived early neural progenitors. As demonstrated by murine models, LHX2 can serve as a selector that plays both active and suppressive roles in cortex formation. However, the role of LHX2 in human neural development is rarely understood. Here, we demonstrated that LHX2 plays a critical role in early neural differentiation of hESCs. Our results revealed that LHX2 was highly expressed proceeded PAX6 and SOX1 and was co-expressed with many neural and post-mitotic neuronal genes. Furthermore, conditional overexpression of LHX2 promoted hESC differentiated toward neural fate whereas disruption of LHX2 by shLHX2 knockdown compromised the neural differentiation of hESC both in vitro and *in vivo*. We further confirmed that LHX2 targeted to PAX6 enhancer and cis-regulated PAX6 promoter by ChIP and reporter analysis. Collectively, our results demonstrate that LHX2 is a key factor for neural fate determination during early pluripotent to neural fate transition.

Poster Board Number: T-3101

ELUCIDATING MOLECULAR FUNCTION DOWNSTREAM OF THE HOMEOBOX PROTEIN HEX USING MOUSE EMBRYONIC STEM CELL MODELS FOR DISTINCT ENDODERMAL PROGENITOR POPULATIONS

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Immediately following the induction of endoderm, regional identity is initially established in the anterior with the expression of the homeodomain Hex. The anterior endoderm is both an important patterning centre and the precursor population for the liver and pancreas. In mammalian development, endoderm formation occurs in two phases and the fate of these populations is different. In the blastocyst, ICM cells generate the primitive endoderm (PrE) which will give rise to the extra-embryonic parietal and visceral endoderm. Hex is initially expressed throughout the PrE and then becomes restricted to the anterior visceral endoderm (AVE), an important embryonic signalling centre. During gastrulation, a second wave of definitive endoderm (DE) differentiation occurs, generating the foregut. One of the earliest specification events in this lineage is also the specification of anterior fate by Hex, this time the anterior definitive endoderm (ADE). We have developed an embryonic stem (ES) cell model to generate either embryonic or extra-embryonic endoderm under defined conditions while monitoring anterior identity. We have used this system, alongside Hex mutant ES cells, to work out the network downstream of anterior induction in both visceral and definitive endoderm. Gene expression analysis on Hex positive populations in ES cell differentiation has been used to characterize key downstream targets and we are beginning functional analysis on these factors. The overall phenotype exhibited in the Hex null ES cells was also assessed by micro-array. This data indicates that Hex null endoderm expresses higher levels of posterior markers, consistent with posterior and non-anterior visceral identity, and consistent with the role of Hex as a key regulator of anterior gene expression. Our data also suggests that one of the key distinguishing characteristics between endodermal populations may be positional markers, rather than their future contribution. Thus, the potency of visceral and definitive endoderm for downstream differentiation may depend on their respective regional identity, rather than their prospective embryonic fate.

Poster Board Number: T-3102

INSULIN-LIKE GROWTH FACTOR 2 ENHANCES CHONDROGENIC DIFFERENTIATION OF PARTHENOGENETIC MURINE EMBRYONIC STEM CELLS UNDER THE THREE DIMENSIONAL CULTURING ENVIRONMENT

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The generation of cartilaginous tissues using pluripotent stem cells can be one of the solutions for cartilage regeneration and skeletal tissue repair, as the mass production of cartilaginous cells is required for the cure of cartilaginous defects and diseases. Parthenogenetic ESCs (PESCs) can be a useful stem cell source. The defects in full-term development of this cell type enable researchers to avoid the ethical concerns. Moreover, in female patients, if the PESCs are derived from her own oocytes, the cells will have that patient's genetic information. Here we presented data demonstrating that PESCs can be differentiated into chondrogenic cells by the induction medium containing multiple factors such as ascorbic acid, dexamethasone, bone morphogenetic protein-2 (Bmp-2), and transforming growth factor-beta (Tgf-β) and this can be promoted by exogenous insulin-like growth factor 2 (Igf2), which gene is silenced in PESCs. Before chondrogenic cell induction, floating aggregates called embryoid bodies (EBs) were formed from the PESCs by suspension culture in the medium containing serum and retinoic acid for 5 days. Then, various concentration of Igf2 supplementation (0, 1, 10 or 100 ng/ml) from the formation of EBs to chondrogenic induction by 3D culture system was investigated. After the induction, gene expressions of chondrocyte specific markers of Chordin-like 1, Collagen-2, Aggrecan, Decorin, MMP13 and Pax-1 were analyzed by real time PCR. As a result, supplementation of 100 ng/ml Igf2 showed the highest capability of chondrogenic induc-

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tion than other groups. The expression levels of all genes were 7 to 35 fold higher in 100 ng/ml Igf2 treated group than Igf2-free control. The results show that supplementation of Igf2 of optimal concentration improves the efficiency of chondrogenic differentiation of murine PESCs under 3D culturing environment. Our finding provides insights into the processes of cartilaginous tissue repair and the imprinting mechanisms active in monogenic stem cells. This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST; Grant number 2011-0027807) and Technology Development Program for Agriculture and Forestry, Ministry for Agriculture, Forestry and Fisheries (MAFF; Grant numbers 109020-3 and 111160-4), Korea.

Poster Board Number: T-3103

THYROID HORMONE PROMOTES DOPAMINERGIC NEURONS DIFFERENTIATION FROM MOUSE EMBRYONIC NEURAL STEM CELLS THROUGH A CALCIUM WAVE DEPENDENT SIGNALING PATHWAY

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Dopaminergic neurons have been implicated in the regulation of motor behavior. Here, we found that hypothyroidism during pregnancy leads to significant decreasing of tyrosine hydroxylase (TH) activity and dopamine neuron loss in the midbrain of the offspring. Results from the open field test carried out when the offspring was 2 month old revealed a significant decrease of locomotor activity of the offspring suffering hypothyroidism during the embryonic phase. Followed, we cultured embryonic neural stem cells (eNSCs) from mouse mesencephalon and treated the cells with a physiological level of T3. We found that T3 treatment significant increase TH gene expression. T3 treatment also increased the number of TH-positive cells after withdrawing the growth factor EGF and bFGF in the culture medium. Further more, we detected the expression of TRPC family members in eNSCs after T3 treatment and found that TRPC1 mRNA and protein levels were remarkably upregulated. In addition, a calcium wave was induced by T3 treatment in cultured eNSCs, and it was significant blocked by SKF96365, an inhibitor of the TRP receptor family. The TRPC1 specific siRNA, which eliminated the TRPC1 expression, was also found to decrease the calcium wave induced by T3. We also found that both SKF96365 and the TRPC1 targeting siRNA eliminated T3-induced upregulation of TH activity and the promotive effects on dopaminergic neurons differentiation. These results suggested that T3 induced dopaminergic neurons differentiation of eNSCs through a calcium wave dependent manner. Taken together, our results reveal the effects of T3 in the dopaminergic neurons generation and explore the underlying mechanisms. This study emphasis the critical effects of T3 in the brain dopamine system development.

Poster Board Number: T-3104

HOXA3 IS ESSENTIAL FOR THE DEVELOPMENT OF THYMIC EPITHELIAL PROGENITORS FROM MURINE EMBRYONIC STEM CELLS

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Hoxa3, a member of the Hox family of transcription factors, has been proposed to be the earliest regulator for thymus organogenesis. Mice homozygous for Hoxa3 deletion ($Hoxa3^{-/-}$) are athymic. Because Hoxa3 is expressed in the pharyngeal pouch endoderm and surrounding neural crest-derived mesenthyme, the absence of a thymus in $Hoxa3^{-/-}$ mice could result from defects in either or both of these tissues, or result from a defect in the third pharyngeal pouch formation. We have reported that murine embryonic stem cells (mESCs) can be induced to generate thymic epithelial progenitors (TEPs) *in vitro*. To determine whether Hoxa3 plays a role in the development of thymic epithelium, we established mESC lines from $Hoxa3^{-/-}$ and the wild-type ($Hoxa3^{+/+}$) mice. We then induced the differentiation of $Hoxa3^{-/-}$ mESCs and $Hoxa3^{+/+}$ mESCs into TEPs *in vitro*. Although $EpCAM1^+$ cells were generated from both $Hoxa3^{-/-}$ and $Hoxa3^{+/+}$ mESCs, $Hoxa3^{+/+}$ mESC-$EpCAM1^+$ cells co-expressed Keratin 5 and Keratin 8, a phenotype of TEPs, whereas $Hoxa3^{-/-}$ mESC-$EpCAM1^+$ cells did not. In addition, $Hoxa3^{+/+}$ mESC-$EpCAM1^+$ cells expressed TEP-related genes Pax1, Pax9, and Plet1 at significantly higher levels than $Hoxa3^{-/-}$ mESC-$EpCAM1^+$ cells. Furthermore, when transplanted under the kidney capsule of syngeneic mice, $Hoxa3^{+/+}$ mESC-$EpCAM1^+$ cells developed into cortical TECs and medullary TECs, reconstituted normal thymic architecture and support T cell development, whereas $Hoxa3^{-/-}$ mESC-$EpCAM1^+$ cells did not. These data suggest that TEPs can be generated from $Hoxa3^{+/+}$ mESCs but not from $Hoxa3^{-/-}$ mESCs. Therefore, Hoxa3 is required for the development of TEPs from mESCs.

Poster Board Number: T-3105

GATA4 COOPERATES WITH NODAL/ACTIVIN SIGNALING DURING DEFINITIVE ENDODERM SPECIFICATION OF MOUSE EMBRYONIC STEM CELLS

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Specification of definitive endoderm (DE) during gastrulation is a prerequisite for the formation of organs including liver, pancreas, lung, thymus, thyroid and gut. DE and mesoderm originate during gastrulation when epiblast cells are recruited to the primitive streak (PS). The PS can be subdivided into posterior, middle, and anterior regions with distinct developmental potential. Definitive endoderm forms from the anterior PS, while distinct subpopulations of mesoderm are induced in each of the different PS regions. These differences in potential are correlated with a gradient of Nodal signaling within the primitive streak. Differentiation of mouse embryonic stem (ES) cells in the presence of Activin A, a nodal receptor agonist, efficiently recapitulates this early lineage commitment: high levels of Activin induce endoderm specification and low levels of Activin induce mesoderm commitment. This co-regulation by Nodal signaling and the close proximity of mesoderm and DE within the PS led to the proposal of a bipotential population known as mesendoderm, now largely supported by experimentation *in vitro* and in the mouse embryo. Nodal/Activin signaling directs the expression of a conserved network of transcription factors that function at different stages in the induction and specification of DE. This network includes Mix-like homeobox genes, Eomesodermin, FoxA2, Sox17 and Gata4/5/6. This network is thought to be involved in segregation of the endoderm and mesoderm lineages, commitment of cells to DE fate, and regionalization of nascent endoderm. To define the role of Gata4 in mouse DE development we generated a mouse ES cell line in which Gata4 is placed under doxycycline-inducible control. We found that in serum-free conditions, Gata4 expression alone favors a primitive endoderm fate, and is not able to induce a DE population. However, expression of Gata4

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under optimal conditions known to favor DE specification (Activin at 75ng/ml) led to a significant increase in the percentage of DE precursors, defined by c-Kit and Cxcr4 expression. More importantly, with Activin concentrations as low as 1 or 5 ng/ml, which is not sufficient to induce DE, Gata4 expression resulted in a large expansion in the DE population. With low Activin, Gata4 directed an increase in the expression of DE specific markers with a concomitant reduction in mesoderm specific markers. This DE population generated by Gata4+low Activin has an equivalent potential for differentiation toward hepatoblast fate, when compared to DE precursors obtained by high Activin concentration. In summary, we show that Gata4 cooperates with Nodal/Activin signaling to specify definitive endoderm and to promote definitive endoderm over mesoderm fate by inducing and reinforcing the expression of endoderm genes, while repressing expression of mesoderm genes. Our results provide the first evidence of a role of Gata4 in the segregation of DE from mesendoderm precursors.

Poster Board Number: T-3106

UNIQUE 3-O-SULFATED HEPARAN SULFATE STRUCTURE CONTRIBUTES TO THE DIFFERENTIATION OF MOUSE EMBRYONIC STEM CELLS

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Recently, we demonstrated that heparan sulfate (HS) chains play important roles in the maintenance and differentiation of mouse embryonic stem cells (mESCs) by regulating extrinsic signaling. Sulfated HS structures are modified by various sulfotransferases during development. However, the significance of specific HS structures during development remains unclear. Here, we show that unique 3-*O*-sulfated HS structures (3-*O*-HS) synthesized by HS 3-*O*-sulfotransferases (3OSTs) increase during differentiation of mESCs following LIF withdrawal. This finding suggested that unique 3-*O*-HS contributes to primary mechanism of differentiation in mESCs. We performed *3OST*-overexpression to up-regulate the unique 3-*O*-HS. Overexpression of the unique 3-*O*-HS in mESCs induced spontaneous differentiation into primitive endoderm and primitive ectoderm in the presence of LIF and serum. To examine the requirement for unique 3-*O*-HS for the differentiation of mESCs, we performed stable knockdown (KD) of *3OST* mRNA using RNAi. In the stable *3OST* KD, the expression of *Gata6* (primitive endoderm marker) did not increase even by LIF withdrawal. Then, we investigated *in vitro* differentiation into embryoid bodies (EBs), which comprise three germ layers: endoderm, mesoderm, and ectoderm. In EBs derived from stable *3OST* KD cells, the expression of *Fgf5* (primitive ectoderm marker), *Sox17* (endoderm marker), Goosecoid (mesoderm marker), and *Pax6* (ectoderm marker) was decreased by down-regulation of the unique 3-*O*-HS. In this study, we demonstrated that the unique 3-*O*-HS structure was necessary for induction of differentiation of mESCs into primitive endoderm and EBs.

Poster Board Number: T-3107

THE DIFFERENTIAL CHARACTERS BETWEEN CRANIOFACIAL AND TRUNK NEURAL CREST DERIVED STEM CELLS BY ANALYSING P0-CRE/FLOXED-EGFP TRANSGENIC MICE

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Neural crest derived stem cells (NCSCs) are migratory cell populations that originate in the dorsal part of the neural tube. After migration, they differentiate into a wide range of tissues, including neurons and glial cells of the peripheral (autonomic and enteric) nervous systems, smooth muscles of the heart and great vessel, bone, cartilage, and connective tissues of the face, and melanocytes. Our purpose was to clarify the differential molecular features of craniofacial versus trunk NCSCs using P0-Cre/Floxed-EGFP mice embryos, in which NCSCs specifically express EGFP. We isolated craniofacial and trunk NCSCs by FACS-purifying EGFP positive cells. Gene expression profiles showed differently up-regulated NCSCs markers between the two regions. These differences showed that craniofacial NCSCs had a mesenchymal lineage, but that trunk NCSCs had a distinct neuronal lineage. These results support previous findings. In addition, we confirmed a number of molecules that were up-regulated in craniofacial NCSCs. These molecules had not previously been reported to be related to craniofacial NCSCs, and might be useful as novel markers for craniofacial NCSCs. In a sphere-forming assay using EGFP positive NCSCs of both regions, NCSCs showed a high capacity for proliferation. Furthermore, through valid differentiation experiments, we confirmed the differentiation potential of a wide range of neural crest derivatives, including neurons, glial cells, adipocytes, cartilage, and bone cells. Comparing the differentiation potential in EGFP positive NCSCs between the two regions, we demonstrated that their differentiation potential was not the same. Our study reports the molecular character difference between craniofacial and trunk NCSCs in P0-Cre/Floxed-EGFP mouse embryos. These data should facilitate efforts in regenerative medicine concerning tissues differentiated from NCSCs. With further investigation of P0-Cre/Floxed-EGFP mice, we can obtain much more helpful findings.

Poster Board Number: T-3108

GLUCOSAMINE INDUCES MIGRATION OF MOUSE EMBRYONIC STEM CELLS THROUGH DISASSOCIATION OF INTEGRIN B4 AND PLECTIN VIA SP1 DEPENDENT-CAM AND PKC SIGNALING PATHWAY

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Glucosamine (GlcN), a naturally occurring amino monosaccharide, is an excellent precursor for the biosynthesis of glycosylated proteins, which is found to modulate cell proliferation, matrix synthesis, and gene expression in various cell types. However, the function of GlcN in mouse embryonic stem cells (mESCs) has not been reported. Therefore, we examined the effect of the GlcN on mESC migration and its related signal pathway. GlcN (2 mM) increased migration after 12 h incubation; this effect was inhibited

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by the GLUT1-specific siRNA. In experiments to examine related signaling pathways, GlcN increased O-linked N-acetylglucosamine (O-GlcNAc) and O-GlcNAc transferase (OGT), but did not elicit any hanges in O-GlcNAcase (OGA) expression. GlcN-induced increase of O-GlcNAc and OGT were inhibited by GLUT1-specific siRNA. Moreover, GlcN induced phosphorylation of specificity protein 1 (Sp1) transcription factor, which was blocked by OGT inhibitor ST045849 (20 μM) in nuclear fraction. GlcN also increased calmodulin (CaM) expression which was blocked by SP1-specific siRNA. In addition, GlcN induced binding of CaM to plectin and then plectin-integrin β4 complex disassembly of HDs, which was reversed by OGT- and SP1-specific siRNA. On the other hand, GlcN increased Ca^{2+} influx and phosphorylation of the protein kinase C (PKC), which were inhibited by GLUT1-specific siRNA or EGTA plus BAPTA-AM. Furthermore, GlcN increased phosphorylation of integrin β4 serine sites which was reversed by PKC inhibitor bisindolymaleimide. In addition, GlcN decreased cell-cell junction regulatory proteins such as ZO-1 and claudin, E-cadherin, and occludin, which were blocked by CaM-specific siRNA or bisindolymaleimide. Finally, GlcN-induced increase of cell migration was inhibited by each signal pathway-related specific siRNA or inhibitors. In conclusion, GlcN regulated dissociation of integrin β4 and plectin through Sp1 dependent-CaM or PKC phosphorylation and thereby stimulating to the mouse ESC migration.

Poster Board Number: T-3109

CONTEXT DEPENDENT ROLE OF OCT4 IN GOVERNING PLURIPOTENCY AND DIFFERENTIATION

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Oct4 is a critical regulator of pluripotency and is indispensible in mouse preimplantation embryo development and embryonic stem cells (ESC) maintenance. In *Oct4* knockout embryos, the inner cell mass cannot be maintained and differentiates into trophectoderm. Likewise, ESC undergo rapid differentiation upon the loss of *Oct4*. However, *Oct4* is not restricted only to naïve pluripotent cells. Rather its expression persists in the postimplantation epiblast and during early differentiation in the gastrulating embryo. While considerable efforts have been dedicated in elucidating factors that are essential for cell fate specification and commitment, the effects of disrupting the tightly regulated pluripotent network during differentiation in response to different environmental signals is still poorly understood. Here, using conditional knockout approaches, we show that *Oct4* is essential in maintaining different states of pluripotency (naïve and primed) in the pre- and postimplantation embryos.

Poster Board Number: T-3110

CONTINUOUS LIVE IMAGING OF SINGLE MOUSE EMBRYONIC STEM CELL CYCLE PROGRESSION USING FUCCI AND E-CADHERIN PROTEINS

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With the emergence of embryonic stem cell-based therapy as an attractive treatment for various diseases, there is a parallel need to analyze cell cycle restructures in differentiation process to assess their therapeutic effectiveness. A comprehensive understanding of the cell biology of embryonic stem cells requires direct observation of single ES cell cycle division in the absence of heterogeneous niche. Here, we combined bio-imaging and cadherin-based ECM engineering approaches to visualize mES cells in a single level. By continuous direct long-term single-cell observation of Fucci mES cells, it was possible to track dynamic cell cycle progression from fast dividing mES up to endoderm lineage-stage cells. With this unique combination, we were able to understand individual cell cycle phase, speed and direction of cell commitment *in vitro*. Abbreviation: mES-mouse embryonic stem cells; ECM- extracellular matrix; Fucci- fluorescent ubiquitination-based cell cycle indicator.

Poster Board Number: T-3111

TEMPORALLY-REGULATED MECHANOTRANSDUCTION CONTROLS STEM CELL CARDIOMYOGENESIS

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As cells migrate and differentiate throughout development, they secrete and assemble extracellular matrix (ECM), giving rise to time-dependent, tissue-specific stiffness, i.e. cardiac muscle originates from soft mesoderm, <500 Pa (Pa; a unit of stiffness), and undergoes ~10-fold myocardial stiffening from HH stages 18-36 in the chick embryo. When mimicked *in vitro*, myocardial stiffening enhanced cardiac specific gene expression and myofibril organization in immature pre-cardiac mesoderm. While active mechanotransduction causes maturation, the specific proteins responsible for responding to time-dependent stiffness involved remain unknown. Here we examined expression and organization of well-known mechanosensitive proteins, e.g. focal adhesion kinase (FAK) and vinculin, in mouse embryonic stem cells (mESCs), mESC-derived cardiomyocytes, pre-cardiac progenitors, and isolated mature cardiomyocytes plated on matrices with either dynamic or static cardiac tissue-specific stiffness. Ratiometric western blot analysis shows higher expression of activated focal adhesion proteins when progenitor and mESC-derived cardiomyocytes were plated on hydrogels of cardiac tissue-specific stiffness compared to static, soft matrices, indicating that mechanically driven maturation is at least partially achieved via active mechanosensing. Beating and force generation were also inhibited in cells on matrices that deviated from tissue stiffness, indicating that active forces required for cell maturation and viability are affected by mechanical properties of the matrix on which the cells exert tension. Identifying mechanosensitive pathways that are active in cardiomyogenesis can lead to a better understanding of stem cell differentiation and development.

Poster Board Number: T-3112

UNDERLYING MECHANISMS OF THE *IN VITRO* INDUCTION OF HEMATOPOIETIC STEM CELL-LIKE CELLS FROM MOUSE EMBRYONIC STEM CELLS BY LIM-HOMEOBOX TRANSCRIPTION FACTOR, LHX2

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We have recently reported that hematopoietic stem cell (HSC)-like cells were effectively induced from mouse embryonic stem cells (ESCs) by an enforced expression of LIM-homeobox transcription factor, *Lhx2* (*Blood* 117:3748-58, 2011). When *Lhx2* was retrovirally-transduced in mesodermal cells of differentiating mouse ESCs

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on OP9 stromal cells, HSC-like cells were induced and robustly expanded. The *Lhx2*-induced HSC-like cells displayed self-renewing capacity *in vitro* and long-term hematopoietic repopulation activity *in vivo* when transplanted into lethally-irradiated congenic recipient mice. The HSC-like cells were also induced from mouse induced pluripotent stem cells. Here we analyzed mechanisms of the *in vitro* induction of HSC-like cells by *Lhx2*. For this purpose, we newly established a mouse ESC line carrying Dox-inducible *Lhx2*. When the ESCs were induced to differentiate on OP9 cells and *Lhx2* expression was turned on at the mesodermal stage, differentiation of lineage$^+$ mature hematopoietic cells from c-Kit$^+$ immature hematopoietic cells was severely impaired. The c-Kit$^+$ cells acquired self-renewal ability and were expanded on OP9 cells. This self-renewal ability strictly depended on the presence of *Lhx2*, since the c-Kit$^+$ cells were immediately differentiated into mature hematopoietic cells after *Lhx2* expression was turned off. These data suggest a possibility that *Lhx2* would promote the self-renewal of immature hematopoietic cells. To confirm this possibility, we carried out the *Lhx2* transduction experiment into mouse adult bone marrow HSCs. *Lhx2* indeed promoted the self-renewal of bone marrow HSCs *in vitro*. Next, molecular functions of Lhx2 were investigated. It has been reported that LIM-homeobox transcription factors indirectly affect the amount of LIM-only protein (Lmo). Consistent with the previous report, we found that in the presence of Lhx2, the amount of Lmo2 protein, a critical regulator of hematopoietic differentiation, was reduced. To determine whether the amount of Lmo2 is a limiting factor during the induction of HSC-like cells by *Lhx2*, *Lmo2* was co-transduced with *Lhx2* in the ESC-derived mesodermal cells. As a result, induction of HSC-like cells by *Lhx2* was partially inhibited by increasing the amount of Lmo2. Taken together, we propose that the induction of ESC-derived HSC-like cells by *Lhx2* is caused by the promotion of self-renewal of resident immature hematopoietic cells, and the amount of Lmo2 is critically involved in this process.

Poster Board Number: T-3113

HEPATIC DIFFERENTIATION OF ES CELLS IN A HOLLOW FIBER-TYPE BIOREACTOR AND APPLICATION TO A BIOARTIFICIAL LIVER

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Introduction: We have tried to develop a bioartificial liver (BAL) using cultured hepatocytes and focused on pluripotent stem cells, such as embryonic stem (ES) cells and induced pluripotent stem (iPS) cells, as a potential cell source of it. These cells must be induced to differentiate into the target cells, hepatocytes, because they are in an undifferentiated state. In this study, we used hollow fiber (HF)/organoid culture method, in which cultured cells form a cellular aggregate called an "organoid" in the lumen of the HF. We have confirmed the hepatic differentiation of mouse pluripotent stem cells using the HF/organoid culture method. To put the BAL using cultured hepatocytes into practical use, it is necessary to develop a high-performance artificial liver device and to demonstrate its therapeutic effect. In the present study, we evaluated the performance of an ES cells-immobilized BAL module based on HF/organoid culture by in vitro and ex vivo experiment. Materials and Methods: [1] Fabrication of the BAL module and hepatic differentiation of mouse ES cells We developed a BAL module (culture volume; 2.97 cm3) with a one-layer textile HF sheet. The HF sheet was comprised of 5 cm, 130 cellulose triacetate HFs for plasma separation. 6.2×106 mouse ES cells were immobilized in the BAL module and the module was cultured under the perfusion condition for 30 days. To induce hepatic differentiation, differentiation-promoting agents were added to the culture medium (1mM sodium butyrate on days 9-15; 10-7 M dexamethasone; 10 ng/mL oncostatin M; and insulin-transferrin-selenium (10 mg/mL insulin, 5.5 mg/mL transferrin, 6.7 ng/mL selenium acid) on days 15-30). [2] Functional evaluation of the BAL module using liver failure model animals Male Wistar rats weighting 200 to 300 g were used for the animal experiments. The liver failure was induced by a method combining 70% partial hepatectomy with hepatic ischemia for 20 min (spontaneous recovery rate; 60%). After the induction of liver failure, the rats were connected with the ES-module or control-module (without cells) via jugular vein and carotid artery, and 1 hour extracorporeal circulation at 1 ml/min was initiated. We measured changes in the blood biochemistry level during extracorporeal circulation. In case the rat recovered, we also evaluated liver regeneration a week after operation. Result: Mouse ES cells immobilized inside HFs proliferated in culture and formed cylindrical organoids that grew in the longitudinal direction of the HFs. The liver specific functions were detected by about 2 weeks of culture. The maximum ammonia removal rate and albumin secretion rate per ES cell-immobilized module reached 60% and same level of that of primary mouse hepatocyte-immobilized module, respectively. In the animal experiments, all of the 3 rats applied the control-module eventually died. On the other hand, the rat applied ES-module, although the number of application has only been one case, recovered after operation and the blood biochemistry levels a week after become equivalent to the healthy level and liver regeneration was also observed. Conclusion: The hollow fiber type BAL module containing differentiating ES cells expressed liver specific functions. In functional evaluation of the BAL module by ex vivo experiments, although it needs further researches, this module showed the potential for supporting liver functions and inducing liver regeneration of the liver failure rats.

Poster Board Number: T-3114

SMALL MOLECULE SIGNALLING PATHWAY INHIBITOR SCREENING REVEALS MULTIPLE PATHWAYS CONTRIBUTE TO DOPAMINERGIC NEUROGENESIS

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Identification of key signalling events during neural differentiation of mouse ES cells (mESCs) has identified the role that some tyrosine kinases play in directing lineage specification. Many of the best defined of current neural differentiation paradigms give rise to either non-desired neural phenotypes, or to non-neuronal cells. The undesired cell types may generate undesired effects if the neurons are to be used for either toxicity, drug screening or transplantation studies. In this study we use homologous knock-in ES cell reporter lines expressing luciferase or eGFP under the control of the transcription factors; Lmx1a, Nurr1 & Pitx3, to investigate the impact of tyrosine kinase inhibitor libraries on the development of dopaminergic neurons. Initial screening used a simple adherent monolayer ES cell differentiation protocol to investigate the effects of 130 kinase inhibitors on Lmx1a-luc expression. From the initial screening, 10 classes of kinase inhibitors were found to consistently influence expression of Lmx1a. In particular, inhibitors of the epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) receptor and

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DNA-dependent protein kinase (DNA-PK) signalling, significantly increased Lmx1a expression (2-, 4- & 2.5-fold change respectively over vehicle, one-way ANOVA, $p<0.0001$). In addition, protein kinase C (PKC) isoform inhibitors generally decreased Lmx1a expression. To confirm small molecule treatment conferred midbrain dopaminergic potential, Pitx3-eGFP cultures were incubated with combinations of EGF receptor, VEGF receptor & PKC inhibitors. The expression of eGFP was then monitored during neuronal development. Under these conditions the attenuation of EGF & VEGF signalling alone was sufficient to promote Pitx3 expression (6.5 ±1.2% & 7.0 ±1.2% vs control 3.5 ±0.4%). In addition, to show small molecule compounds also increased midbrain dopaminergic precursors, Nurr1-luc cultures were exposed to candidate inhibitors. However, unlike Lmx1a, candidates could not modulate expression of Nurr1. This work shows that small molecule screening reveals select pathways that stimulate or inhibit transcription factors associated with the development of dopaminergic neurons without the use of exogenous patterning factors. However, the timing of inhibitor addition & potential for signalling pathway cross-talk may confound our interpretation of this data; both factors are currently under investigation.

Poster Board Number: T-3115

MICRORNA125B-LIN28 PATHWAY ACTS AS A RHEOSTAT TO CONTROL THE BALANCE BETWEEN SELF-RENEWAL AND MESENDODERM COMMITMENT OF EMBRYONIC STEM CELLS

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Embryonic stem cells (ESCs), derived from the inner cell mass of blastocysts, are self-renewing and pluripotent cells with the ability to differentiate into all derivatives of the three primary germ layers, i.e. ectoderm, endoderm, and mesoderm. However, the factors and signaling processes regulating self-renewal and differentiation are not yet fully understood. MicroRNAs (miRNAs), small noncoding RNAs that regulate gene expression at the posttranscriptional level, are important regulators of cell fate decisions. Although thousands of miRNAs and their targets have been identified, their specific roles in regulating the balance between self-renewal and specific lineage commitment of ESCs are largely unidentified. Here we report a novel rheostat function of miR-125b/Lin28 axis in the regulation of mouse embryonic stem cell (mESC) self-renewal and lineage specification. With a MicroRNA Array screen, we identified a number of miRNAs significantly changed during ESC differentiation, among which miR-125b was enriched in undifferentiated ESCs but more significantly downregulated than most other miRNAs during early differentiation. The abundantly expressed miR-125b in undifferentiated mESCs was dramatically downregulated to a level hardly detected during differentiation day 3 to 5, with a concomitant upregulation of Lin28. Ectopically expressing miR-125b did not alter characteristics of undifferentiated mESCs, whereas it sustained the cells in the undifferentiated status, impaired the endoderm and mesoderm development and inhibited cardiomyocyte formation, while the expression of ectodermal genes and the neuronal differentiation remained unchanged. We further demonstrated that miR-125b targeted the 3'-untranslated region of Lin28 and reduced the abundance of Lin28 at both mRNA and protein levels. Moreover, phenotypes of miR-125b overexpressing cells were mimicked by downregulation of Lin28 and rescued by re-introduction of Lin28. In addition, the impaired cardiogenesis in miR-125b introduced cells was greatly recovered when mimicking of endoderm environment by cultivation with the condition medium from a visceral endoderm-like cell line END-2. These results demonstrate that the downregulation of miR-125b is required for the initiation of ESC differentiation and miR-125b acts as a rheostat that controls the balance between self-renewal and lineage-specific commitment of ESCs through directly targeting Lin28. These findings extend our knowledge in understanding of the regulatory mechanism of ESC self-renewal and differentiation as well as the tightly control of cell lineage decisions in ESCs.

Poster Board Number: T-3116

APPLICATION OF STEM CELL BASED SCREENING TECHNOLOGY TO COSMETIC DEVELOPMENT

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Background and Purpose: The appearance of human skin is dramatically transformed as we age. The signs of skin aging include pigmentation, wrinkles, sagging skin, dry skin and gray hair. These signs are considered to be induced when the maintenance of tissue homeostasis is disrupted. Recent studies have shown that stem cells exist in skin tissues such as epidermis, dermis and subcutaneous tissues, and may play an important role in the maintenance of tissues. We have pursued the development of novel cosmetic technologies targeting skin stem cells (Biochem Biophys Res Commun. 396, 837-842. 2010). In this study, we discussed the pathogenic mechanism of pigmentation and gray hair and the screening technology for materials to improve them, focusing on melanocyte stem cells locating in the bulge area in hair follicles. Specifically, we established an *in vitro* stem cell differentiation induction system for melanocytes and conducted a search for materials that regulate the differentiation of melanocytes using the system. Methods: In this study, ES cell (C57BL/6-derived) differentiation induction system for melanocytes established by Yamane et al. was used (Dev Dyn.216, 450-458. 1999). First, ES cells were seeded on the ST2 feeder layer and were induced to differentiate into melanocytes using a culture medium (α-MEM) in which FBS (fetal bovine serum), DEX (dexamethasone), bFGF (basic fibroblast growth factor), CT (cholera toxin) and EDN3 (endothelin-3) were added. Then, a variety of materials was added in the differentiation induction system. And the effects of each material on the differentiation into melanocytes were examined by analyzing the gene expressions of differentiation markers Results: As a result of the screening using the differentiation induction system, we discovered various materials which regulate the differentiation of ES cells into melanocytes. For example, barley (gramineous grain) extract notably promoted the differentiation into melanocytes, but in contrast, *Alaria Praelonga* (seaweed) extract notably suppressed the differentiation into melanocytes. We also found a material that altered ES cell fate and promoted the ES cells to differentiate into nerve cells and retinal pigment cells. We will further investigate these materials and analyze the regulatory mechanism of stem cell differentiation. Discussion: The materials discovered in the screening system are considered to be versatile. For example, barely extract, which promoted the differentiation into melanocytes, may be useful for the prevention of gray hair and vitiligo which is caused by loss of melanocytes. On the other hand, *Alaria Praelonga* extract, which suppressed the differentiation into melanocytes, may be useful for the treatment of pigmentation, dullness and hyperpigmentation of the skin. Furthermore, the materials, which promoted the differentiation into nerve cells and retinal pigment cells, can be applied to the treatment of neurological diseases such as Parkinson's disease and retinal pigmentary dystrophy. It is expected that the stem cell-based screening technology is a useful means of providing a number of information on abilities of screened materials. Currently, the identification of active

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ingredients, analysis of regulatory mechanism of differentiation and consideration of the clinical efficacy of the materials in which we found a regulatory effect of stem cell differentiation are being conducted.

Poster Board Number: T-3117

MODULATING CELL FATE AND LINEAGE COMMITMENT VIA MRNAS ENCODING CELL TYPE-SPECIFIC TRANSCRIPTION FACTORS

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The ability to manipulate cell fate offers tremendous potential as a tool for understanding lineage commitment and differentiation during development, and as a tool for *in vitro* disease modeling or direct therapeutic applications. Currently, two main strategies are employed *in vitro* to direct cell fate: (1) directed differentiation, in which pluripotent stem cells are guided through a series of steps that mimic development *in vivo*, and (2) direct lineage reprogramming or transdifferentiation, in which one fully differentiated cell type is converted into another. Multiple examples of direct lineage reprogramming through forced expression of cell-type specific transcription factors have now been reported, including those of the skeletal muscle, neuronal, cardiac, and pancreatic lineages. Current methods primarily rely on viral-mediated gene delivery, which presents risks associated with genomic integration and insertional mutagenesis that limit translational applications. Furthermore, these methods do not allow for stoichiometric and temporal control over factor delivery. Here, we present the introduction of transcription factors via transfection of non-integrating mRNAs for direct modulation of cell fate. Using the master myogenic transcription factor, *MyoD*, we present proof-of-concept on the utility of non-integrative mRNA-based methods for the transdifferentiation of mouse and human fibroblasts into skeletal muscle. We show that (1) MyoD mRNA is efficiently translated into protein following transfection (2) MyoD protein is functional in activating a MyoD-sensitive luciferase reporter, and (3) repeated delivery of MyoD mRNA to fibroblasts induces expression of the muscle-specific markers, MF-20, Myogenin, Desmin, as well as the formation of multinucleated myotubes.

Poster Board Number: T-3118

CELLULAR IDENTITIES ARISING DURING MESODERMAL DIFFERENTIATION

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Differentiation of multi- or pluri-potent cells results in the generation of heterogeneity of cell identities in a poorly understood manner. Understanding the nature in which the heterogeneity emerges at the cellular level during differentiation is essential to allowing an understanding of the mechanisms that control this process. This is partly because measurements on heterogeneous populations cannot confirm correlations at the individual cell level, but more importantly because the generation of heterogeneity is the primary output of the differentiation process. The generation of diversity can be considered as the passage of cells (and their descendants) through an N-dimensional descriptive space (i.e. phase space) where each dimension represents some numerical descriptor of the cell state. We are using combinatorial oligonucleotide fluorescent *in situ* hybridisation (FISH) to quantitatively measure transcript abundancies for up to 7 genes concurrently during mesoderm differentiation. This allows us to track the occupancy of cell identities in N-dimensional (N <= 7) phase space, and to monitor how this occupancy changes with time or change in conditions. We are initially interested in the extent of the allowable space (a reflection of the rigidity of the controlling mechanisms), and how dependent this is on external and internal conditions. In order examine this we are making measurements from large numbers (>1000) of individual cells at different times and conditions during the differentation of mouse ES derived mesoderm progenitors to hematopoietic and endothelial lineages. This should provide, not only an idea as to the general nature of the process but also an extensive data set that can provide answers to a range of specific questions and issues. Finally, we hope that this data set, in combination with data directly specifying interactions between components can be combined to construct an explanatory model of the controlling mechanisms.

Poster Board Number: T-3119

DEFINING OSTEOGENESIS TO ELUCIDATE BONE REPAIR MECHANISMS DURING THE NORMAL AND DISEASED STATE

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Osteoporosis is a skeletal degenerative disease which disproportionately affects a large number of postmenopasual women, but can also afflict a smaller percentage of men. This disease is characterized by low bone mineral density (BMD) leading to bone fragility fractures and bone structural deterioration caused by the imbalance between bone resorption by osteoclasts and bone formation by osteoblasts. In postmenopausal osteoporosis disease models, it has been found that bone forming osteoblast cell production is reduced, while bone resorption osteoclast cells remain functioning. Additionally, osteoblasts produced from sources such as bone marrow derived mesenchymal stem cells (BMSCs) is reduced with advancing age. The ability to create a source of osteoblasts would be ideal to restore balance to bone resorption and formation, thus cure the root of this degenerative disease. Our preliminary results demonstrate that we have generated a robust and easy to culture human embryonic progenitor (hEP) cell lines derived from human embryonic stem (hES) cells. To determine the capability of these cells to become osteoblasts, a small scale screen of the hEPs was used to evaluate cell morphology and mineralization for osteogenic properties. Under osteogenic differentiation conditions, a subset of the hEP cells displayed mineralization as detected by Alzarian red and Von Kossa staining. The osteogenesic potential of the hEP cell lines after 21 days in osteogenic differentiation media was further confirmed by examining gene expression patterns for the osteogenic differentiation markers runx2/cbfa1 (Runx2), and osteocalcin (BGLAP). Interestingly, the phenotype of the BMSCs differed from the osteoprogenitor hEP lines under bone forming conditions. The osteoprogenitor hEPs formed only osteoblasts, however, no chondrogenic and adipogenic differentiation was observed with these cells when cultured under adipogenic, chondrogenic, or osteogenic differentiation media. We have elucidated that different cell signaling pathways are being suppressed and activated in the osteoporgenitor hEPs as compared to the BMSCs. Thus we have identified osteoprogenitor cell lines which may be useful as a surrogate model to study osteoblast differentiation and be used as a potential regenerative therapy for osteoporosis.

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Poster Board Number: T-3120

DERIVATION OF NEURAL STEM CELLS FROM EPIBLAST STEM CELLS

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Pluripotent stem cells can be derived from both pre- and post-implantation embryos. Embryonic stem cells (ES cells), derived from blastocyst are naïve pluripotent and epiblast stem cells (EpiSCs) derived from post-implantation epiblast are primed pluripotent. The phenotypes and gene expression patterns of the two pluripotent stem cells are different each other and EpiSCs thought to be in a more advanced pluripotent (primed pluripotent state) than mouse ES cells (aïve pluripotent state). Therefore, we questioned whether EpiSCs are less potential to be differentiated into specialized cell types *in vitro*. We derived EpiSCs from 5.5-6.5 day post coitum mouse embryos. The EpiSCs could differentiate into all three germ layers *in vitro*, and expressed pluripotency markers. Interestingly, EpiSCs also were able to efficiently differentiate into neural stem cells (NSCs). The NSCs differentiated from EpiSCs (EpiSC-NSCs) expressed NSC markers (Nestin, Sox2, and Musasi), self-renewed over passage 20, and could differentiate into three neural subtypes, neurons, astrocytes, and oligodendrocytes. Next, we compared global gene expression patterns of EpiSC-NSCs with that of NSCs differentiated from ES cells and brain tissue. Gene expression pattern of brain tissue derived NSCs were closer to ES cell-derived NSCs than EpiSC-NSCs, indicating that the pluripotent stem cell-derived somatic cells could have different characteristics depending on the origin of pluripotent stem cell types.

Poster Board Number: T-3121

NEUROTROPHIN SYSTEM: A NEW MOLECULAR PLAYER DURING VASCULAR DIFFERENTIATION OF EMBRYONIC STEM CELLS

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Embryonic stem cells (ESCs) have the ability to self-renew indefinitely and to differentiate in any bodily cell type, including vascular cells. Hence, ESC-bases therapies represent a big hope for promoting vascular repair in ischemic disease. Neurotrophins (NTs) are a family of protein ligands (NGF, BDNF, NT4/5 and NT3) which mediate their action by binding tropomyosin receptor kinases (TRKA, B, C). Well known for their actions on the nervous system, NTs were more recently recognized to promote angiogenesis and regulate stem cell biology, including of ESCs. Here, we analyzed the role of NTs during vascular differentiation of ESCs (mouse ES-D3 line) into vascular endothelial cells (ECs) and smooth muscle cells (SMCs). Using a 2D embryoid bodies (EBs) model of differentiation, which enables production of both ECs and SMCs (Jakobsson et al, 2007), we first analyzed the mRNA expressional changes in pluripotency and vascular markers, and in NTs and TRKs during differentiation. Pluripotency markers (Nanog, Oct4) halved expression at day 7 of EB-vascular differentiation. Early in EB development, at day 4, mRNA level of SMC markers increased (αSMA: 7 folds, SM22α: 2 folds vs pluripotency) while EC markers mRNA were enhanced from day 7 (CD31: 5 folds, CD144: 4 folds vs pluripotency). Among the NT system, BDNF/TRKB and NT3/TRKC were the most expressed during EB formation. They displayed a differential mRNA expression pattern (qRT-PCR), with an earlier increase of NT3 and its preferential TRKC receptor (8 and 20 folds vs pluripotency, respectively) at day 4, followed by a later increase of BDNF and its TRKB receptor (10 and 4.5 folds vs pluripotency, respectively) near day 7. VEGF (VEGF-A_{165}, 20ng/ml) is known to induce the expansion of EBs to a peripheral vascular plexus (Jakobsson et al, 2007). We confirmed this data (by immunofluorescence) and discovered that, importantly, recombinant BDNF or NT3 (25ng/ml for both protein) stimulated a comparable organized vascular structure, composed of mural cells surrounding endothelial tubes. Moreover, BDNF increased mRNA level of both EC and SMC markers; while NT-3 enhanced SMC markers, only. The vascular structures were higher and thicker by treating EBs with VEGF (10ng/ml) plus either BDNF or NT3 (12.5ng/ml), thus suggesting synergies between VEGFs and NTs. Inhibition of signalling through either TRKB (small non peptidic compound ANA-12, 25mM) or TRKC (soluble TrkC receptor domain TrkCd5 neutralizing NT3, 2µg/ml) leaded to the formation of a vascular plexus less structured. Interestingly, mRNA levels of both EC and SMC markers were decreased under ANA-12 treatment, although only mRNA of SMC markers was affected by the inhibition of TRKC pathway. These results would suggest a preferential effect of NT3 into the muscular commitment while BDNF would act in both EC and SMC differentiation. In addition to the hereby described role in vascular differentiation, NTs seemed to promote EB expansion. By counting cells at day 10, a crucial time point during EB development, we found significantly higher number of EB cells after VEGF, BDNF or NT3 in comparison with vehicle. This effect was enhanced by the synergic effect of VEGF with either BDNF or NT3. Moreover, ANA-12 and TrkCd5 inhibited cell growth, and so EB expansion. Taken all together, these preliminary data evidence for the first time a role for the NT system in the differentiation process of ESCs into vascular cells.

Poster Board Number: T-3122

DERIVATION OF A NOVEL EPIBLAST STEM CELLS FROM SOMATIC EPIBLAST CELLS

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Since ES cell derivation by Evans and Kaufman, several pluripotent stem cell types have been reported, such as embryonic germ (EG) cells, FAB-stem cells, and epiblast stem cells (EpiSCs). Among these, EpiSCs are classified as "primed" pluripotent stem cells to compare with naïve pluripotent stem cells, ES cells. Epiblast cells differentiated into three germ layers during *in vivo* development. Although EpiSCs contribute to three germ layers in in vitro differentiation, they rarely incorporate into ICM of blastocyst and rarely contribute to chimera after blastocyst injection. However, EpiSCs are very heterogeneous population and not well characterized. Here, we established an EpiSC line that can form chimera efficiently. We derived EpiSCs from Oct4-GFP transgenic mouse epiblast, which express GFP by control of Oct4 regulatory region including promoter, distal enhancer (DE), and proximal enhancer (PE). We derived epiblast stem cells from embryonic day 6.5 embryos. All epiblast stem cell lines expressed GFP and self-renewed over 50 passages. Newly derived EpiSCs expressed pluripotency markers Oct4 and Nanog, but did not express germline markers, which were known to be expressed in EpiSCs before; so we called these somatic EpiSCs (sEpiSCs). The sEpiSCs were able to differentiate to all three germ layers and formed teratoma and chimeric embryos without germ cell contribution. sEpiSCs showed completely different gene expression pattern from ES cells and E3 EpiSC line. Therefore, we derived a novel pluripotent cell type that is different from ES cells and EpiSCs. The sEpiSCs could be derived from somatic epiblast-

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specific cells, which had been separated from specialized epiblast cells that have a developmental potential into primordial germ cells.

Poster Board Number: T-3123

CONTROLLED OXYGEN MARKEDLY INFLUENCES DIFFERENTIATION OF EMBRYONIC STEM CELLS TO INSULIN PRODUCING CELLS

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Pluripotent stem cells (PSC) hold promise for cell replacement therapy and studying embryonic development. However, efficient differentiation to desired cell types remains a major obstacle. Most PSC research is performed in high, non-physiological O2, but cells during embryonic development are exposed to much lower O2. Here we report a wide-ranging study showing that physiological O2 markedly influences differentiation to insulin-producing cells. We differentiated human embryonic stem cells (hESC) and under different, well-characterized pO2 environments, controlling cellular oxygen exposure through adhesion culture on highly O2-permeable silicone rubber membranes. We focused our differentiation of hESC to c-peptide+ cells using a modification of the published 5 stage protocol by ViaCyte, Inc (San Diego, CA)(D'Amour 2006 Nature biotech). We found that differentiation under 5% O2 from hESC to definitive endoderm (stage 1), primitive gut tube (stage 2), and posterior foregut (stage 3), followed by 20% O2 to pancreatic endoderm (stage 4) and insulin-producing cells (stage 5) gives rise to a cell population that is 25% positive for both c-peptide and insulin. This result was 5 times greater when compared to differentiation under normoxic conditions (20% O2). The cells cultured under hypoxia passively secreted c-peptide into the medium but were not glucose responsive. All pancreatic endoderm genetic markers were increased for the controlled-hypoxia (5% stage 1-3, 20% stage 4-5) when compared to the normoxia condition (20% stage 1-5). By examining each stage at multiple controlled high and low oxygen levels, we identified O2 conditions that increased the fraction of the intermediate cell type of each cell measured by flow cytometry, or increased expression of genetic markers for those intermediate stages measured by real-time PCR. By combining these oxygen levels appropriately, we achieved a large improvement. Preliminary results on stage 4 cells have indicated potential O2 conditions that result in a 50% c-peptide+ population. Based on these results O2 combined with other directed differentiation protocols is a potentially-straightforward method that could be applied to future cell therapy protocols to generate more of a desired cell type.

Poster Board Number: T-3124

AN ES CELL-DIFFERENTIATION SYSTEM RECAPITULATES A DEVELOPMENTALLY REGULATED NEURON-SPECIFIC PARENT-OF-ORIGIN EXPRESSIVITY

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Genomic imprinting is a phenomenon where mono-allelic gene expression is observed in a parent-of-origin manner. A subset of the imprinted genes acquires a tissue-specific imprinted status during the course of tissue development but mechanisms underlying this epigenetic regulation were largely elusive. We sought to investigate this process of tissue-specific genomic imprinting by utilizing an in vitro differentiation system of embryonic stem (ES) cells. Ube3a gene is expressed from the maternal allele only in neurons. Judging from its expression pattern during development, the involvement of the paternally-expressed non-coding antisense RNA in Ube3a imprinting was anticipated. We established hybrid ES cells between two sub-species of Mus musculus (C57BL/6 and MSM/Ms) useful to study genomic imprinting mechanism because of the abundant inter-subspecies SNPs, and succeeded in the establishment of an in vitro neuronal differentiation system where neuron-specific imprinting of Ube3a is recapitulated. With this system, we revealed that the switch from bi-allelic expression to maternal mono-allelic expression of Ube3a occurs during the late neuronal development, neurite outgrowth period, and that the expression of endogenous antisense transcript of Ube3a locus is up-regulated several hundred-fold at the same period. By analyzing hybrid mouse embryos, we also confirmed Ube3a mono-allelic expressivity from embryonic day 15.5 in the developing nervous system. Concomitantly, marked Ube3a-antisense transcriptional up-regulation was also observed, confirming the "epigenetically" faithful differentiation of our hybrid ES cells. Preliminary in situ hybridization studies independently detecting sense and antisense strands showed that the Ube3a-AS transcripts were mostly observed within the cell nuclei of the neural cells. This suggests a cis-acting mechanism of these antisense transcripts. Further studies are under way to elucidate the precise role of these non-coding RNAs. An important implication from this study is that evaluation of the "quality" of the cells derived from in vitro differentiation of ES cells needs scrutinizing epigenetic aspects such as genomic imprinting status found in tissues *in vivo*, in addition to the evaluation by differentiation gene markers and morphology. These hybrid ES cells and in vitro differentiation system will also allow researchers to investigate broader analyses of neuron-specific genomic imprinting, and our hybrid ES cells can be useful resources for other tissue-specific genomic imprinting and epigenetic analyses.

Poster Board Number: T-3125

MECHANISTIC INSIGHTS INTO DIDO3 FUNCTION IN STEM CELLS

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The *death inducer-obliterator* (*Dido)* gene has been identified in genome-wide screens as a potential regulator of embryonic stem cell pluripotency. To explore the role in stem cell biology of Dido3, the largest and most broadly expressed of the three Dido isoforms, we generated embryonic stem cells from a Dido3-specific mouse mutant, using red fluorescence protein (RFP) to replace the only *Dido3*-specific exon in the *Dido* locus (Dido3-*delta*CT(C-terminal)), that shows impeded epiblast cell differentiation at the onset of gastrulation, causing death at embryonic day 8.5. Functional analysis of mutant ES cells showed indefinite self-renewal capacity, but indicated that Dido3 is necessary for stem cell differentiation in response to withdrawal of leukemia inhibitory factor (LIF). Wild type (wt) Dido3 protein is highly expressed in wt ES cells and is downregulated during differentiation. Mutated Dido3-*delta*CT is expressed at comparable levels in mutant ES cells, but is not downregulated during differentiation. Other stemness-related genes showed impaired repression in mutant embryonic bodies (EB), particularly Oct4, a gatekeeper for ES cell differentiation; mutant EB also showed impaired upregulation of genes necessary for differentiation. To study the mechanism of Dido3 function, we stably overexpressed distinct Dido3 protein domains in mutant

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Dido3-*delta*CT ES cells and monitored expression of stemness and differentiation genes in the EB. Reconstitution with full-length Dido3 restored differentiation capacity in Dido3-*delta*CT mutants, with downregulation of Dido3 itself and of other stemness genes, including Oct4, and upregulation of differentiation genes. Overexpression of the CT domain, missing in the Dido3-*delta*CT mutant, triggered upregulation of differentiation genes, but not downregulation of stemness genes. In contrast, overexpression of the NT domain (N-terminal common region of the three Dido isoforms) resulted in rapid downregulation of stemness genes and upregulation of differentiation genes. Further experiments revealed Dido3 interaction with the HDAC3-SMRT complex, as well as Wwp2 and RNA polymerase II, suggesting a role in transcriptional repression. Based on these results, we speculate that Dido3 is involved in repression of differentiation genes in stem cells, as well as in correct downregulation of stemness genes at the onset of differentiation.

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NOVEL EPIGENETIC REGULATORY MECHANISM OF OCT4 BY CDK2AP1 IN ESCS

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Understanding the key regulatory elements in maintenance of stem cell pluripotency is fundamental in using stem cells in clinical applications. Cell cycle regulators are gaining a more prominent role, these include molecules such as p27 that have been shown to play a role in cell cycle kinetics to maintain a pluripotent state and regulate specific genes involved in pluripotency. Similarly, our group has discovered how a novel molecule in cell cycle known as Cyclin Dependent Kinase 2 Associated Protein 1 (CDK2AP1) is involved with key aspects of pluripotency and differentiation. Knockdown of CDK2AP1 in mice leads to an embryonically lethal phenotype, suggesting a critical role in development. Furthermore, mouse embryonic stem cells with CDK2AP1 deletion remain pluripotent with removal of LIF when compared to WT. One possible mechanism is the association of CDK2AP1 with the nucleosome remodeling and deacetylase (NuRD) complex. We have shown that CDK2AP1 interacts with HDAC2 and MBD3, two major players in the NuRD complex. Deletion of CDK2AP1 in mESC shows reduced levels of global DNA methylation and increased levels of global histone acetylation. Recently we have shown CDK2AP1 to regulate *Oct4* expression, a master regulator in stem cell renewal and differentiation. In our studies we have shown that CDK2AP1 plays a key role in NuRD-mediated *Oct3/4* silencing by epigenetically regulating the *Oct4* promoter during differentiation of both mESC and hESC. Detailed analysis of the *Oct4* promoter revealed an absence of DNA methylation at the proximal enhancer (PE) region in differentiated *Cdk2ap1*$^{-/-}$ mESC. In parallel, we have seen an increase in H3K9 acetylation at the same region in *Cdk2ap1*$^{-/-}$ mESC. We have found CDK2AP1 occupancy at the PE region in mESC as well as hESC embryoid bodies. Furthermore, in mESC we have observed interdependency in CDK2AP1 and MBD3 binding to the OCT4 promoter. In hESC nuclear translocation of CDK2AP1 upon differentiation was distinct from mESC. CDK2AP1 plays a significant role in stem cell differentiation by association with the NuRD complex on specific promoter regions, changing chromatin accessibility and leading to the silencing of the *Oct4* promoter during differentiation in mESC. CDK2AP1 itself behaves differently from our IF experiments between human and mouse ESC. We are currently delineating the mechanistic role of CDK2AP1 in hESC, as well as differences and similarities with mESC.

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EPO PREVENT BLOOD-RETINAL BARRIER IN DR THROUGH RECUITMENT OF BMPS

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Background: Retinal ischemia induced eye diseases such as Diabetic Retinopathy (DR) and Retinopathy of Prematurity (ROP) are the leading cause of blindness in the world. An inadequate blood supply resulting from early vessel loss causes tissue hypoxia, which determines the severity of subsequent pathological vessel growth and the rest usually cause vision loss by abnormal blood vessel growth. Antiangiogenic compounds (VEGF antibody) often fail to completely inhibit abnormal vascular growth and are associated with underlying complications. Since the potential clinical application, any effective treatment for preotection retinal vessel would be benificial to patients which were suffered by these diseases. Methods: Firstly, we used intraperitoneal injection STZ to induce early DR and OIR (Oxygen-induced retinopathy) to induce ROP animal model. The mechanism of prevention effect to blood-retinal barrier (BRB) of EPO were studied; Secondly, using lentiviral mediated system to up-regulate or suppress EPO expression level in early DR and ROP animal, we monitored the process of these diseases. Furthermore, using bone marrow transplantation (BMT) and intravitreal injection bone marrow derived progenitor (BMP), we studied the EPO protection mechanism in histological level, which could analyze the vessel cells quantity change of BRB in DR and ROP. Thirdly, after interference of EPO receptor expression by siRNA, we studied the EPO relative signaling pathway in vivo and in vitro. Results: In our study, we found that EPO could prevent the injured BRB in early DR and ROP. EPO also could preserve the DR and ROP retinal apoptosis and electrophysiology function. In retinal flat mount and dissociated retinal FCM experiments, we found that all three cell types containing in the BRB were significantly rescued by EPO gene therapy. In the way of recruit BMP, EPO could improve the BRB morphology and function in both early DR and ROP. In vivo and in vitro studies shown that these EPO specific effects were mediated by its receptors which could activate the downstream signaling pathway. Conclusions: Thus, we will hypothesize that EPO could be serving as a BMP chemoattractant, which can recruited BMP mediated by EPOR and CD131. We found that EPO could recruit BMP migration to the injured retina and maintain vascular hemodynamic stability. The molecular mechanism of these protection effects was further investigated lentiviral mediated siRNA to block the specific receptors. In this study we also found BMP behavior is highly dependent on nitric oxide (NO). In conclusion, it is suggestion that the promising goal of retinopathy treatment is to rebuild and stabilize functional vasculature in hypoxic retinal tissue, rather than to eliminate the abnormal vessels. Furthermore, the advantages of possible cytokine therapy are suitable in wide-use regardless of the underlining genetic defect.

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MULTIPLE ANALYSES OF G-PROTEIN COUPLED RECEPTOR EXPRESSION IN THE NEURAL DIFFERENTIATION FROM EMBRYONIC STEM CELLS

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Embryonic stem cells (ES cells) will be valuable resources for clinical therapies because of their unlimited self-renewal ability and potential to generate any differentiated cell type. G protein coupled receptors (GPCRs) play key role in many complex biological processes, including development. However, the role of GPCRs in ES cell pluripotency and differentiation has received little attention. We demonstrated the role of GPCRs on mouse ES cells differentiation including neural or glial differentiation from neural stem cells, and pluripotency. Adrenergic receptor alpha 1a, alpha 2a and alpha 2c were upregulated with the progress of the differentiation from ES cell, whereas adrenergic receptor beta 3 (Adrb3) was dramatically decreased with the progress of the differentiation from ES cell into neural stem cells. The change in the histone modification at the promoter region of Abrb3 was seen in the neural stem cell development from ES cells. Under these conditions, a significant increase in lysine 27 on histone H3 (H3K27) trimethylation at the promoter of Adrb3 was observed in the primary neurosphere derived from ES cells. These results suggest that changes in adrenergic receptors signaling pathway along with epigenetic modification may a play a role in maintaining ES cells.

Reprogramming

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DIRECT GENERATION OF NEURAL PRECURSOR-LIKE CELLS FROM ADULT HUMAN DERMAL FIBROBLASTS.

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Recent studies have reported direct reprogramming of fibroblasts to mature neurons by the introduction of defined neural genes. This technology has potential use in the areas of neurological disease modelling and drug development. However, the use of induced neurons for large-scale drug screening and cell-based replacement strategies is limited due to their inability to expand once reprogrammed. We propose it would be more desirable to induce expandable neural precursor (iNP) cells directly from fibroblasts. In support of this proposal, we have demonstrated the ability to transform adult human dermal fibroblasts directly into neural precursor-like (iNP) cells. This was achieved by forced expression of the neural stem cell factors Sox2 and Pax6 using either nonviral plasmid transfection or recombinant protein transduction. Further, we have optimized cell culture conditions allowing us to generate iNP colonies in the absence of an animal or human feeder cell layer, an essential requirement for the transfer of reprogrammed cell lines to clinical use. Colony formation was observed within 14 - 30 days with full colony expansion achieved by 60 - 90 days at an efficiency of 0.05%. Daughter colonies were formed after serial passaging. Quantitative PCR demonstrated that iNP colonies express a range of neural stem and precursor genes including Sox2, Pax6, Six3, Sox1, HoxB9, Nkx6 and Ngn2, comparable to human embryonic stem cell-derived neural precursor cells. Upon differentiation, iNP cells give rise to neurons exhibiting typical neuronal morphologies including multiple arborizing dendrites and expression of the neuronal markers TUJ-1, NSE and MAP2, with a population of iNP-derived neurons expressing the catecholaminergic neuronal marker TH or the GABAergic neuronal marker $GAD_{65/67.}$ Most importantly, iNP-derived neurons demonstrated the ability to fire action potentials in response to current injection. These results represent a novel virus-free approach for direct reprogramming of human fibroblasts to a neural precursor fate.

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REPROGRAMMING OF HUMAN NEUROBLASTOMA CELLS USING IPSC TECHNOLOGY

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Background: Neuroblastoma (NB) is the most common extracranial solid tumor of infancy, which originated from the sympathetic nervous system of the neural crest. Oncogene MYCN amplification is frequently observed and is associated with the aggressiveness of NB. Our recent studies provide novel stratifications of NB which are based on global genomic and RNA expression profiles. These molecular signatures are strongly correlated with patient prognosis, suggesting that each subtype has a specific course of progression. Induced pluripotent stem cells (iPSCs) can be generated from distinct cell types by enforced expression of reprogramming factor (RPF) genes. Recently, induced pluripotent cancer cells (iPCCs) have been established in several cancers and are being used to investigate the stemness of cancer cells. However, generation of iPCCs from NB has never been attempted. Here we aimed to generate and profound study of iPCCs from NB. Methods: We applied the Sendai virus (SeV) vector into SH-SY5Y I-type NB cells for reprogramming, as it is an RNA virus and transgenes do not integrate into the host genome. We carried out 244K human array CGH (aCGH) microarray to examine the gene expression profile of iPCCs. We performed alkaline phosphatase (AP) staining to confirm iPCC generation. To examine the embryonic stem (ES) cell-related genes expressed in iPCCs, immunocytochemistry and quantitative real time RT-PCR were performed. Apoptotic cells were detected using TUNEL assay after treatment with chemotherapeutic agents. Results: SH-SY5Y intermediate (I) type cells show higher expression of RPF genes such as NANOG, OCT4, SOX-2, KLF4 and have stem-like properties. Upon retinoic acid (RA) treatment they undergo differentiatiation into neuroblastic (N) and substrate adherent (S) type cells. Forty days post transduction of SeV, NB cells form iPS-like colonies and AP staining positive data confirm iPCCs generation. The aCGH data revealed the same genomic aberrations in the iPCCs as their parental cells, indicating that an intact genomic status was maintained during the reprogramming process. Immunocytochemistry data suggested that the iPCCs expressed ES cell surface markers such as SSEA4, TRA-1-60 and TRA-1-81. Quantitative RT-PCR data revealed that endogenous reprogramming factor genes including NANOG, OCT4, SOX2, c-MYC and hTERT were highly

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expressed in these colonies. Cis-diamminedichloroplatinum (CDDP) treatment to iPCCs showed a resistant phenotype when compared with their parental cells. Conclusions: We generated iPCCs from human NB which show cancer stem cell (CSCs)-like properties that might create a new opportunity for disease modeling, patient-specific drug screening and personalized cell-based therapies.

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UNDERSTANDING REPROGRAMMING USING THE X CHROMOSOME REACTIVATION

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X chromosome inactivation is an exemplar for epigenetic inheritance. Reactivation of the X inactive chromosome (Xi) can occur both in vivo and in vitro when female somatic cells acquire a pluripotent state, and this provides a model to study the erasure of epigenetic memory in mouse cells. In human, however, the status of the two X chromosomes in female embryonic stem (ES) cells and their reactivation in induced Pluripotent Stem (iPS) cells varies depending upon culturing and reprogramming techniques. The aim of our research is to understand how human somatic cells are reprogrammed towards pluripotency, by examining the X chromosome reactivation in heterokaryons where human fibroblasts or lymphocytes are reprogrammed following fusion with mouse ES cells. This methodology, unlike iPS, allows monitoring early events that occur during successful reprogramming. We have examined known epigenetic marks of the inactive X chromosome during reprogramming and defined the sequence of events that are associated with the X reactivation in the human nuclei, such as chromatin features, XIST localization and biallelic expression of X-linked genes. Delocalization of XIST and loss of H3K27me3 enrichment from the Barr body are some of the first events that occurs concomitantly with *de novo* expression of pluripotency genes. Sequential RNA/DNA-FISH data suggest that this is followed by a reactivation of at least some X-linked genes. Future studies will extend these findings analyzing the expression of X-linked polymorphisms after reprogramming of clonal fibroblast populations by both RFLP and Allele-specific RNA sequencing approaches. This study will provide insights into the status of the X chromosomes in reprogrammed human cells and will help us to untie the controversial results obtained with the iPS method thus furthering our understanding of stem cell identity.

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LARGE SCALE ANALYSES OF MOLECULAR SIGNATURES AND BEHAVIORS OF HUMAN PLURIPOTENT STEM CELLS

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There are some variations in differentiation propensity among clones of human embryonic stem (hES) cells and induced pluripotent stem (hiPS) cells. For the promising applications of ES/iPS cells: drug discovery, studies on disease mechanism and cell therapy, we need to select the clones capable of complete differentiation into expected cells with no remaining of undifferentiated cells, preferably without differentiation processes, which are time- and cost- consuming. We established 49 hiPS cells from some types of cells of donors with various age and sex by various methods. And we examined the gene/miRNA expression and methylation status of these and compared with those of 10 hES cells. As a result, we demonstrated that molecular signatures of hES cells and hiPS cells are very similar although some variations exist. To determine whether these variations are related to the differentiation propensity into neural cells or not, we performed neural induction for 3 hESCs and 40 hiPSCs by using modified serum-free floating culture of embryoid body-like aggregates (SFEBq) methods and examined the percentage of not only the early neural marker, PSA-NCAM positive cells, but also the undifferentiated marker, OCT3/4 positive cells after neural induction. All the tested clones differentiated into the PSA-NCAM positive cells with high efficiency (>80%), but some clones showed more than 10% remaining of OCT3/4 positive cells in at least one experiment. These clones formed teratoma when transplanted into striata of NOD/Scid mice brains after terminal differentiation, so we defined these clones as "bad" clones. Moreover, by analysis for gene/miRNA expressions of "good" and "bad" clones before differentiation, we found some molecular factors by which we can predict clones with/without remaining undifferentiated cells after neural differentiation. Combination of these factors will help to delete the "bad" clones which have possibility to form teratoma after transplantation and not suitable for clinical application.

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ACTIVIN AND POLO-LIKE KINASE I SIGNALING REGULATE THE CONVERSION OF MOUSE AND HUMAN CELLS INTO INDUCED NEURONS

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The mammalian nervous system comprises many distinct neuronal subtypes, each with its own phenotype and differential sensitivity to degenerative disease. Although specific neuronal types can be isolated from rodents or engineered from stem cells for translational studies, transcription factor mediated reprogramming might provide a more direct route to their generation. Recent studies have demonstrated that the forced expression of select transcription factors is sufficient to convert mouse and human fibroblasts and stem cells directly into a variety of neuronal subtypes. However, the utility of this approach is currently limited by the low efficiency of conversion. One potential solution is to identify small molecules that increase induced neuron generation. Such chemicals would enable the generation of large numbers of patient-specific neurons for disease studies and provide insight into the mechanisms that regulate neuronal induction by defined factors. To this end, we used a functional reprogramming screen to identify small molecules that increase the rate of transcription factor-mediated conversion of mouse adult fibroblasts into *Hb9*::GFP+ spinal motor neurons. An inhibitor of Activin-like kinases 4/5/7 and a Polo-like kinase I (PLK1) inhibitor each increased induced motor neuron formation by 5-10-fold. In combination, the chemicals increased the rate of induced motor neuron formation by 50-fold. Both small molecules also increased the rate of conversion of human fibroblasts and embryonic stem cells into motor neurons, indicating that these chemicals should enable the generation of human patient-specific motor neurons for disease modeling. After using peptide or small molecule analogues of both chemicals to confirm that Activin and PLK1 signaling are the functional targets of the these molecules during motor neuron induction, we performed pulse treatments at different times during reprogramming to determine when they are most effective. We found that the Activin inhibitor was effective when administered from days 1-5, 6-10, or 11-15. In contrast, the PLK1 in-

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hibitor was only active during days 6-10, suggesting it acts on transient intermediates in the cultures. These results suggest that these chemicals act by divergent mechanisms. Because Activin inhibition was effective even after many motor neurons had appeared, we hypothesized that it might enhance motor neuron survival. Indeed, we found that chemical treatment greatly promoted the survival of flow-purified mouse and human motor neurons in culture, indicating that Activin inhibition can act by promoting neuronal survival. To determine if Activin inhibition increases conversion into other neuronal types, we transduced fibroblasts with *Ascl1*, *Myt1l*, and *Brn2*, transcription factors that induce the formation of non-motor neurons, and cultured the cells with or without the Activin inhibitor. Chemical treatment increased the number of neurons generated by 10-fold, indicating this approach may be applicable to a variety of neuronal types. We have identified small molecules that increase the rate of direct conversion of mouse and human fibroblasts and stem cells into motor neurons. These results identify the Activin and Polo-like kinase I signaling pathways as major roadblocks to induced neuron formation and indicate that many neurons are lost shortly after conversion. Finally, these chemicals should enable the efficient generation of induced neurons for patient-specific disease modeling.

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EFFICIENT GENERATION OF TRANSGENE-FREE HUMAN AND MOUSE IPS CELLS USING A CELL-PERMEANT TAT-CRE PROTEIN

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Reprogramming of somatic cells using viral transduction of defined transcription factors remains a widely used and efficient method to obtain induced pluripotent stem cells (iPSCs). However, the presence of viral transgenes in iPSC is undesirable as it raises the possibility of insertional mutagenesis leading to malignant transformation and has also been shown to affect differentiation potential. Various strategies have been employed to address this issue, including non-integrating viruses, RNA transfection, protein transduction and site specific recombinases to excise the transgenes after reprogramming. Here we show efficient generation of transgene-free mouse and human iPSCs through the use of a Cre-excisable polycistronic lentiviral vector expressing the "stem cell cassette" (STEMCCA) comprised of all four transcription factors (OKSM) followed by exposure of the full reprogrammed iPSC to cell permeable TAT-Cre recombinant protein. Notably highly efficient excision (100% for mouse iPSCs and 60 - 80% for human iPSCs) could be demonstrated following exposure of iPSCs to 4 - 6 µM TAT-Cre for 1 - 2 hours. The high degree of efficiency obtained with protein transduction is in marked contrast to results obtained with electroporation of a plasmid expressing Cre-recombinase (<10%) and also for adenovirus expressing Cre recombinase which has been shown to be effective for mouse iPSCs but not for human iPSCs. Additionally, we present a simple and robust PCR strategy that enables fast identification of deleted clones directly from primary iPSC colonies. Establishment of transgene-free iPSCs required approximately two weeks from the time of addition of the cell-permeant TAT-Cre protein. Factor-free human and mouse iPSCs expressed appropriate morphological and immunochemical staining characteristics of pluripotent cells. Factor-free human iPSCs possessed a normal karyotype and were capable of differentiating into derivatives of all three germ layers *in vivo*. In summary we have established a robust system for highly efficient excision of viral vectors from iPSCs using cell permeant TAT-Cre protein. Efficient delivery of an active recombinant Cre protein to mammalian cells has broad applications not only for somatic cell reprogramming, but also for controlled genetic modification of mammalian genomes.

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ENGINEERING THE REPROGRAMMING OF HUMAN AND MOUSE CELLS TOWARDS THE CARDIAC CELL LINEAGE

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In recent years various groups have successfully demonstrated the transdifferentiation of terminally differentiated cells into alternative cell lineages. In one such case overexpression of MyoD is sufficient to efficiently convert fibroblasts into functional skeletal myocytes. Recent evidence has suggested that this may also be possible for the cardiac cell lineages. The main objective of this work was the identification of a core set of master regulator genes that would activate the efficient transdifferentiation of both human and mouse cells into the cardiac muscle cell lineage. We begun by identifying specific transcription factors (TF) previously shown to actively control cardiogenesis during development (FHL2, GATA4, GATA5, HAND1, HAND2, HEY1, HEY2, HOP, IRX4, MEF2C, Mesp1, MYOCD, NKX2-5, SMARCD3, SRF, TBX5, and TBX20), and subsequently cloning them into an inducible (Tet-On) lentiviral expression vector. To efficiently screen for cardiac reprogramming we utilized lentiviral expression vectors or plasmid DNA molecules allowing the expression of marker proteins under the control of cardiac specific promoters (MYH6pr) in addition to isolating embryonic fibroblasts from transgenic mice (Myh6.GFP.Myh6.PAC). We subsequently transduced human and mouse primary cells (human dermal fibroblasts, human adipose derived cells, and wild type or transgenic mouse embryonic fibroblasts), as well as established cell lines (NIH3T3 and HELA) with various combinations of the identified transcription factors. We screened for potential transdifferentiation events by cell-specific fluorescent protein (GFP, RFP) expression analysis (FACS and microscopy), RT-PCR analysis, immunofluorescence staining, calcium transient detection (GCaMP3) and electrophysiological cell characterization.
One-week post induction of TF transgene expression we readily detected GFP+ or RFP+ cells in samples with particular TF combinations, indicating successful binding and activation of the cardiac specific promoter element. We also measured (qRT-PCR) a significant upregulation of cardiac specific genes including Actc1, Myh6, Myh7, Myl2, Myl7, Nppa, Ryr2, Tnnt2, Casq2, Hcn4, and Pln. Cells also stained positive in a cross-striated pattern when using cardiac specific antibodies (αActinin or Tnnt2). Although no spontaneous contracting was detected, when GCaMP3+ cells were co-cultured with neonatal rat ventricular myocytes we readily detected GFP flashing. We conclude that overexpression of a particular set of TFs can successfully initiate the epigenetic reprogramming towards the cardiac cell lineage.

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DYNAMICS OF LYSINE ACETYLATION DURING THE ONE-CELL STAGE MOUSE EMBRYOS AFTER SOMATIC-CELL NUCLEAR TRANSFER

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Although somatic-cell nuclear transfer (SCNT) is a powerful tool for reprogramming differentiated nuclei into totipotency, the developmental rate has been inefficient as other reprogramming technologies of somatic cells like iPS technology. Treating cloned embryos with trichostatin A (TSA), a HDAC inhibitor (HDACi) has been found to significantly improve their subsequent development. (Kishigami et al. 2006). However, the mechanism for requirement of HDACi in SCNT is not truly understood yet. In pre-implantation embryos, regulation of lysine acetylation through HDAC activity plays a pivotal role in the subsequent embryonic development rates. Further, TSA treatment can induce hyperacetylation of histone and non-histone proteins. To elucidate the mechanism underlying the impact of TSA treatment, we analyzed behaviors of lysine acetylation in fertilized, parthenogenetic and cloned embryos by immunofluorescence. We found that following oocyte activation, lysine-acetylation was increased in both pronuclei and cytoplasm. The presence of TSA significantly enhances lysine-acetylation in the whole cell, suggesting that TSA impact on not only histone but also non-histone protein in cytoplasm. In the pseudo-pronuclei of SCNT embryos, the amount of lysine acetylation was lower than in pronuclei of parthenogenetic embryos, which is consistent with previous reports showing lower histone acetylation in SCNT embryos. Interestingly, lysine-acetylation in the cytoplasm of SCNT embryos was also significantly lower than parthenogenetic embryos, suggesting that somatic-cell injection can induce hypoacetylation. Thus, somatic-cell nuclear transfer impacts on the dynamics of lysine acetylation in embryos, which may contribute to lower developmental potential in SCNT embryos in addition to abnormal gene expression in cloned embryos.

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COMPREHENSIVE ANALYSES OF CHIMERAS AND PROGENY MICE FROM INDUCED PLURIPOTENT STEM CELLS

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Induced pluripotent stem (iPS) cells are generated from somatic cells by introducing small sets of transcription factors. Patients- or disease-specific iPS cells may provide unprecedented resources for drug discovery, studies on disease mechanisms and cell transplantation therapies. Although several assays *in vivo* and in vitro show that mouse and human iPS cells are similar to embryonic stem (ES) cells in many aspects, their long-term behavior and safeness *in vivo* remains to be determined. Moreover, up to the present, diverse modified technologies to generate iPS cells have been developed. However it is still unclear which of them are preferable for applications. Chimera formation by injection of cells into the early embryo is one the most stringent assay to evaluate pluripotency and safeness of mouse iPS cells. In this study, we performed comprehensive analyses of chimeras and progeny mice derived from iPS cells generated with various origins and methods in our laboratory. We herein evaluated 533 chimeric mice and their 484 progenies derived from various iPS cells and 136 several types of control mice. 97% of them were observed until they died. Even among control groups we found significant difference in lifespan. Most chimeras derived from iPS cells generated by the retroviral transduction of Oct3/4, Sox2, and Klf4 with or without L-Myc survived for more than 80 weeks, which are comparable to control mice groups. In contrast, c-Myc integration resulted in a higher incidence of tumors and mortality in both the chimeras and progeny mice. Usage of cMyc with non-integration technology did not show such effect. These data demonstrated that three factor-derived iPS cells, even with proviral integration, are free from either genetic or epigenetic abnormalities which would lead to a shorter longevity in chimeras.

Poster Board Number: T-3140

DIRECT REPROGRAMMING OF MOUSE GALL BLADDER CELLS INTO TRANSPLANTABLE INSULIN-POSTIVE BETA-LIKE CELLS

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Current cell therapies for type 1 diabetes are restricted to transplantation of cadaveric donor islets, but success has been hindered by both lack of donor islets available and by the need for long-term immune suppression in the patient. We hypothesized that reprogrammed gall bladder cells (GBCs) could be used as an autologous cell therapy for type 1 diabetes. Here we show that mouse GBCs can be reprogrammed into transplantable insulin-positive cells using a unique combination of genetic factors and small molecules. First, we show that murine GBCs can be robustly expanded *in vitro*, allowing the generation of billions of cells from a single gall bladder. These GBCs can be transduced by recombinant adenovirus, although optimal transduction requires complexing of the viral particles with DEAE-Dextran. We determined the combination of *Neurog3*, *Pdx1* and *MafA* to be the minimal required transcription factors for robust expression of both *Ins1* and *Ins2* mRNA *in vitro*. We also established that addition of retinoic acid and inhibition of notch signaling increased the reprogramming efficiency of GBCs into beta-like cells. Next, using flow cytometry to isolate reprogrammed cells, we confirmed reprogrammed GBCs were differentiating towards the beta cell fate by both RNA and protein analyses of various transcription factors, including *Nkx2-2*, *Nkx6-1*, *Pax4*, *Pax6*, *Isl1*, and *Neurod1*. Importantly, these reprogrammed cells also began to lose their parent gall bladder phenotype based on decreased expression of many genes, including *Muc1*, *Aqp1*, *Cftr* and *Krt19*. However, although the reprogrammed GBCs were able to produce, process and secrete insulin, they do so in a non-glucose responsive manner. Subsequently, in order to determine the global expression profiles of these cells, we performed RNA-Sequencing analysis to compare reprogrammed GBCs to control GBCs, as well as actual pancreatic beta cells. Interestingly, the analysis confirmed that the reprogrammed GBCs were an intermediate population between gall bladder epithelial and mature pancreatic beta cells. However, reprogrammed GBCs had significant gene expression differences compared to beta cells, and we have identified several key components of beta cell function that are not properly expressed, including genes involved in glucose sensing, insulin vesicle maturation and insulin exocytosis. Finally, these partially reprogrammed

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cells were transplanted subrenally into diabetic NOD-*Rag1*^{null} *IL2r*^{null} *Ins2*^{Akita} (NRG-Akita) and NOD-*scid IL2r*^{null} *Ins2*^{Akita} (NSG-Akita) mice. Importantly, the cells were able to survive long term in NRG-Akita and NSG-Akita mice and were insulin-positive for at least 12 weeks post-transplantation.
Overall then, we hypothesize that reprogramming to the beta cell fate is achievable by expression of *Neurog3*, *Pdx1* and *MafA*. However, additional factors will be required to differentiate these cells *in vitro* into mature, glucose-responsive beta cells. Therefore, we are currently investigating whether additional transcription factors and modulation of specific signaling pathways can achieve reprogramming of GBCs into mature, functional beta cells for the purpose of restoring euglycemia in type 1 diabetes.

Poster Board Number: T-3141

THE EFFECTS OF OCT4 VIRAL VECTOR DELIVERY TO MOUSE BRAIN ON PLURIPOTENCY MARKERS' EXPRESSION

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Introduction: There are several reports of somatic cell reprogramming into pluripotent stem cells (iPSCs) with a combination of four transcription factors, Oct4/Sox2/Klf4/c-Myc. Interestingly, NSCs endogenously express Sox2, c-Myc, and Klf4, so they were reprogrammed into iPSCs just with Oct4 although the efficiency was low. Because of the neural stem cell restriction, brain repair is limited. So increasing neural stem cell number or their potencies could be useful in brain repair. Methods: Oct4 lentiviral vector was injected into the lateral ventricle of C57/BL6 mice brain followed by administration of doxycycline (3 µg/ml) for 5 consequent days. Animals were decapitated at day 7 and total RNA was extracted from the tissue collected from the rims of lateral ventricles. After cDNA synthesis, real time PCR performed to analyze the expression of Oct4, Sox2, Nanog, Klf4, c-Myc, alkaline phosphatase, Sox1and Pax6. Gene expression level was normalized to GAPDH as an internal standard. Moreover, some of the brains were fixed and processed for analyzing expression of Oct4 and Nanog proteins in addition to SSEA1 using immunohistochemistry.
Results: Endogenous expression of Klf4, Nanog, c-Myc, Sox1 and Pax6 mRNAs were increased significantly 7 days after administration of Oct4 lentiviral vector. In addition, increased levels of Nanog and SSEA1 were detected in the brain sections, although Oct4 expression was very low. Conclusion: Exogenous expression of Oct4 vector can induce cells reside in the rim of lateral ventricles to express genes activated in the pluripotency state. This can improve neural stem cell's potencies in SVZ to repair brain degenerations more efficiently.

Poster Board Number: T-3143

THE ROLE OF MICRORNAS IN REPROGRAMMING OF SOMATIC CELLS INTO MOUSE INDUCED PLURIPOTENT STEM CELLS

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The reprogramming of adult somatic cells into induced pluripotent stem cells (iPSCs) holds great promise in patient-specific cell therapy and model for drug screening. iPSCs can be generated by somatic cells nuclear transfer (SCNT) or introduction of Oct-4, Sox2, Klf4, c-Myc (OSKM) into somatic cells. However, the clinical application of iPSCs was impeded by the low reprogramming efficiency. In recent years, there is interest on studying the epigenetic modifications during reprogramming. For instance, treatment with compounds affecting epigenetic regulators such as DNA methyltransferase inhibitors and histone deacetylase (HDAC) inhibitors increased the reprogramming efficiency. On the other hand, the ESC- specific microRNAs (miRNAs) such as the miR-290 cluster were found to promote reprogramming. Our laboratory had determined the miRNA profile of mouse oocytes and preimplantation embryos. We found that some mouse ESC-specific miRNAs and some miRNAs known to be involved in mesenchymal epithelial transition (MET) were highly expressed in 1-cell mouse zygotes. We hypothesized that the miRNAs present in the zygotes regulate epigenetic modulators and thereby facilitating nuclear reprogramming. Therefore, a secondary mouse embryonic fibroblasts (MEFs) system carrying doxycycline inducible transcription factors OSKM was used to study the action of some of these miRNAs on reprogramming. Among the selected miRNAs tested, overexpression of miR-101 and miR-135a inhibited doxycyclin-induced reprogramming by almost 5-fold and 2-fold respectively. On the other hand, inhibition of miR-135a expression enhanced the reprogramming efficiency by around 2-fold; while inhibition of miR-101 expression did not affect reprogramming. In silico analysis predicted Enhancer of zeste homolog 2 (Ezh2) and Sirtuin-1 (Sirt-1) were potential targets of miR-101 and miR-135a, respectively. Both Ezh2 and Sirt-1 were highly expressed in mouse embryonic stem cells (mESs), and their expressions decreased time-dependently during in-vitro differentiation of mESC. On the other hand, the expression of Ezh2 and Sirt-1 increased during the reprogramming of MEFs. Interestingly, our data also showed that knockdown of Ezh2 and Sirt-1 expression significant inhibited the reprogramming efficiency, consistent with the inhibitory effects of miR-101 and miR-135a precursors. The identification of the role of miRNAs in reprogramming could help our understanding of the molecular mechanisms of reprogramming.

Poster Board Number: T-3144

PLURIPOTENTIAL REPROGRAMMING AND RE-DIFFERENTIATION RESET GENOMIC IMPRINTING PATTERNS

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Induced pluripotent stem cells (iPSCs), generated by the overexpression of transcription factors Oct4, Sox2, Klf4 and c-Myc in somatic cells, have the same characteristics as pluripotent embryonic stem cells (ESCs). iPSCs reprogrammed from differentiated cells get through a epigenetic modification during reprogramming and finally have the similar epigenetic state to ESCs. In this study, these epigenetic changes were observed in reprogramming of uni-parental parthenogenetic somatic cells. Furthermore, we have shown that parthenogenetic pattern of imprinted genes were changed during pluripotential reprogramming. Parthenogenetic neural stem cells (pNSCs) containing only maternal alleles regain the biparental imprinting patterns after reprogramming. However, we have yet to define whether the changed imprinted genes are maintained or reverted to the parthenogenetic state when the reprogrammed cells are differentiated into specialized cell types. To address this question, we compared genome-wide expression profiles of biparental female neural stem cells (fNSCs), parthenogenetic neural stem cells (pNSCs), and NSCs differentiated from parthenogenetic maternal iPSC (miPS-NSCs). Furthermore, this study establishes the correla-

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tion between the alteration of genome methylation and activation of imprinting genes in the parthenogenetic cells and reports for the first time that the silenced PWS-related imprinted genes are activated in miPS-NSCs. Our data demonstrated that pluripotential reprogramming of parthenogenetic somatic cells were able to reset the parthenogenetic imprinting patterns; reprogrammed miPSCs showed erasure of maternal methylation imprints and acquisition of methylation in paternally imprinted genes. Furthermore, the changed imprinting patterns were maintained when the reprogrammed cells are differentiated into specialized cell type.

Poster Board Number: T-3145

FACTORS MEDIATING EPITHELIAL-MESENCHYMAL TRANSITION ENHANCE REPROGRAMMING OF SOMATIC CELLS TO PLURIPOTENCY

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Somatic cells can be reprogrammed to pluripotency by the expression of four factors: Oct4, Sox2, Klf4, and c-Myc. This process generally requires weeks and is highly inefficient, and has been shown to involve a mesenchymal to epithelial transition (MET). In order to better understand the mechanism of reprogramming, toward the goal of improving its efficiency, we studied the role of factors mediating epithelial to mesenchymal transition (EMT) during reprogramming of mouse and human cells. Knockdown of Twist or Snail in reprogramming of mouse or human fibroblasts decreases efficiency, while overexpression of these factors via ER fusion in both fibroblasts and keratinocytes improves reprogramming efficiency. Utilizing cells from mice expressing a knockin Snail- or Twist-IRES-YFP, sorted YFP positive populations reprogram at higher efficiency than negative. We observe nuclear Twist in both fibroblasts and keratinocytes early in reprogramming. Reprogramming efficiency varies across mouse strains, and in strains that reprogram more efficiently, the proportion of cells with nuclear Twist is higher. qPCR on mouse and human samples collected during the course of reprogramming reveals expected increases in pluripotency factors, but surprisingly the expression of Snail and Twist increases early in reprogramming in both mesenchymal and epithelial starting cells, before decreasing to the very low levels seen in iPS cells. Our results indicate that Snail and Twist both enhance the efficiency of reprogramming, and that even epithelial cell populations express mesenchymal factors during reprogramming, implying a pre-MET step. Our results suggest that expression of EMT factors is involved in the creation of a reprogramming-amenable state.

Poster Board Number: T-3146

A ROLE FOR ACTIVATION-INDUCED CYTIDINE DEAMINASE (AID) IN STABILIZING INDUCED PLURIPOTENCY

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Reprogramming of a somatic cell by ectopic expression of reprogramming factors to yield induced pluripotent stem cells (iPSCs) has the potential to provide personalized regenerative cell therapies and to model diseases "in a dish". Despite its enormous potential, the generation of iPSCs is a slow asynchronous process with a very low efficiency (<0.1%). One proposed road block to efficiency is incomplete DNA demethylation of pluripotency genes, thereby inhibiting their activation during the process of reprogramming. Activation-induced cytidine deaminase (AID) is primarily known for its role in the generation of antibody diversity in B-lymphocytes. Recently it has been shown that AID can play a role in active DNA demethylation during reprogramming by cellular fusion of mammalian somatic cells. Here we explored the role of AID in direct reprogramming of somatic cells by induction of four factors *(Oct4, cMYC, KLF4 and Sox2)*. Using a polycistronic lentiviral cassette, we expressed the four reprogramming factors in tail tip fibroblasts or embryonic fibroblasts derived from *AID*$^{-/-}$ mice or littermate controls. We observed that initial stages of reprogramming, measured by the expression of SSEA1 and E-CADHERIN, are functional and even enhanced in *AID*$^{-/-}$ fibroblasts. The total number of primary colonies obtained by the reprogramming process is significantly enhanced in knockout fibroblasts ($p<0.001$) compared to the controls, yet clear differences in the morphology of the colonies are observed. The colonies derived from *AID*$^{-/-}$ mice display a flatter morphology lacking the sharp edges normally observed in colonies derived from wildtype mice or embryonic stem (ES) cells, and they are relatively heterogeneous with respect to cell size. We also observed that *AID* expression is induced in wildtype cells transitioning to the iPSC state. We analyzed the expression of core pluripotency markers (SSEA1, NAGOG and OCT4) at different time points during reprogramming, and observed no difference in the frequency of cells expressing these markers comparing *AID*$^{-/-}$ and wildtype derivatives three weeks after induction with reprogramming factors. Furthermore, no differences were observed in the expression of pluripotency markers *Utf1, Col6a2, Thy1, Utf1, Eras, Klf2, Gbx2, Dpp4 and Tbp,* as measured by qRT-PCR. In contrast, 4 weeks after reprogramming was initiated, there is a sharp decline in the frequency of cells expressing pluripotency markers in the *AID*$^{-/-}$ compared to wildtype derivatives ($p<0.001$ for SSEA1 and NANOG and $p<0.01$ for Oct-4). When colonies that appeared morphologically to be pluripotent after three weeks of reprogramming were picked from *AID*$^{-/-}$ derivatives, approximately 40% differentiated during passaging. The other colonies remained stably pluripotent, as measured by their ability to form embryoid bodies and to differentiate into derivatives representing all three primary germ layers. In contrast, all the wildtype-derived colonies remained pluripotent through passage 50. In conclusion, AID plays a role in regulating the process of induced pluripotency, as shown by lower efficiency of complete reprogramming in *AID* knockout cells. *AID* is not essential, and its function may perhaps be compensated by other deaminases. However, in its absence, reprogramming factors generate poorly reprogrammed iPSC colonies, many of which are morphologically and genetically unstable.

Poster Board Number: T-3147

CELLULAR MEMORY DISC OF THE REPROGRAMMING CELLS

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The crucial fact underlying the low efficiency of cellular reprogramming is poorly understood. The cellular reprogramming occurs in nuclear transfer, induced pluripotent stem cell (iPSC), cell fusion, and lineage-switching experiments. Despite these advances, there are three fundamental problems to be addressed: (1) a majority of cells cannot be reprogrammed, (2) the efficiency of the reprogrammed cells is low, and (3) the developed reprogrammed cells

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from patient's own cells activate immunogenic responses. These obstacles present major shortcomings for using reprogramming approaches for customized cell therapy. The current hypothesis is that all cells undergo an endogenous and/or exogenous holographic memorization that parts of it dramatically decrease efficiency of the reprogrammed cells, act like a barrier against reprogramming in the majority of cells, and activate immunogenic responses. Accordingly, the focus of this review was mainly to describe the topic of the cellular memory disc. Based on the present theory of memory disc, the cellular memory includes three parts: reprogramming-resistance memory (RRM), switch-promoting memory (SPM), and cultured induced memory (CIM), which effect on the cellular behaviors.

Poster Board Number: T-3148

P27KIP1 REGULATES SOX2 FUNCTION IN STEM CELLS,DEVELOPMENT AND DIFFERENTIATION THROUGH DIRECT TRANSCRIPTIONAL REPRESSION

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The mechanisms involved in the reprogramming of differentiated cells into induced pluripotent stem (iPS) cells by the three transcription factors Oct4, Klf4 and Sox2 remain poorly understood. Recently, we and others have shown that tumor suppressor genes oppose reprogramming, limiting the efficiency of the process. This barrier against reprogramming could potentially represent an antitumor action of these genes and investigating the interplay between tumor suppressors and genes with important functions in the biology of stem cells might provide clues about the molecular mechanisms governing cancer development as well as increasing our understanding of the reprogramming process. While investigating the activity of different tumor suppressor genes during reprogramming, we realized that cells lacking p27Kip1 could be reprogrammed at low but reproducible frequency with only two factors, Oct4 and Klf4. Mouse embryonic fibroblasts derived from p27Kip1-null animals showed an intrinsically high basal level of expression of Sox2. This un-repressed expression of Sox2 correlated with the absence of repressive chromatin marks at the critical Sox2-SRR2 enhancer upstream of Sox2 gene. Moreover, pluripotent stem cells lacking p27Kip1 fail to fully repress Sox2 upon differentiation. At a mechanistic level, we found that upon differentiation, p27Kip1 directly binds to the critical Sox2-SRR2 enhancer together with a p130-E2F4-Sin3a repressive complex and contributes to transcriptional repression of Sox2.

Poster Board Number: T-3149

TRANSDIFFERENTIATION BY BACTERIAL DELIVERY OF MYOD PROTEIN

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Forced exogenous gene expression has been well characterized as an effective method for directing both cellular differentiation and dedifferentiation. However, transgene expression is not amenable for therapeutic application due to the potential for insertional mutagenesis. Protein based techniques provide a safe alternative, but current protein delivery methods are quite limited by labor-intensive purification processes, low protein yield and inefficient intracellular targeting. Such limitations may be overcome by using a naturally occurring bacterial protein injection system. Pseudomonas aeruginosa utilizes a Type III Secretion System (T3SS) to inject bacterial proteins directly into the eukaryotic cell cytoplasm. Our previous studies describe the ability of this system to easily deliver a high quantity of protein to both differentiated and pluripotent cells using a genetically attenuated strain. Using Cre recombinase as a reporter, we have demonstrated high frequency loxP mediated recombination in the chromosome of the recipient cells, suggesting the protein is not only efficiently targeted to the nucleus, but also retains its biological function. MyoD is a key muscle regulatory factor, the over-expression of which is able to induce transdifferentiation of numerous cell types, such as fibroblasts, into functional myocytes. Here we demonstrate transient injection of MyoD protein by P. aeruginosa to be sufficient to induce myogenic conversion of mouse embryonic fibroblasts. In addition to clear morphological changes, muscle specific gene expression has been observed by both immunostaining as well as RT-PCR. These studies serve as a foundation for the bacterial delivery of transcription factors to efficiently modulate concentration-dependent and temporal activation of gene expression to direct cell fate without jeopardizing genomic integrity.

Poster Board Number: T-3150

DIRECT REPROGRAMMING OF PRIMARY LEUKEMIC CELLS INTO IPS CELLS

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The reprogramming of differentiated cells into induced pluripotent stem cells (iPSCs) by introduction of the four defined transcription factors (Oct4, Sox2, Klf4, and Myc) has rapidly become an intensively investigated area in stem cell research due to its enormous potential in regenerative medicine. Noticeably, like malignant transformation, the generation of iPSCs has also been shown to be suppressed by both the p53 and Rb tumor suppressor pathways, indicating that there are some shared mechanisms between tumorigenesis and iPS reprograming. However, it has not been definitively demonstrated whether or not primary transformed cells (not established tumor cell lines) can be reprogrammed into iPSCs with a full-term developmental potential in mice. To this end, we established acute myeloid leukemia (AML) by over-expressing the leukemogenic fusion protein, MLL-AF9, in bone marrow-derived hematopoietic cells from doxycycline (Dox)-inducible transgenic mice in which expression of the four reprograming factors can be turned on by Dox in culture (Brambrink et al.,2008). The sorted leukemic cells were then induced into iPSCs by addition of Dox under mouse embryonic stem cell (ESC) culture conditions. By using genomic PCR, we confirmed that the *MLL-AF9* fusion gene was present in the iPS colonies. However, our RT-PCR result indicated the leukemic gene was silent in the iPSCs. The characteristics of these iPS cell lines were similar to those of normal ESCs, as assessed by the expression of pluripotency genes and the presence of iPSC surface markers. The karyotypes of all tested iPSC lines from the leukemic samples were normal, with 40 chromosomes each. All of these iPSC lines could form teratomas and differentiate into three germ layers. More importantly, the iPSC lines were able to generate chimeric mice with a high integrating rate. In short, our current

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study demonstrates that despite the presence of a leukemogenic gene, the primary leukemic cells could be directly converted into iPSC lines *in vitro* and the MLL-AF9 carrying iPSCs were able to contribute to chimeras in mice. We are currently investigating whether leukemia-derived iPSCs have a full-term developmental potential with germ-line transmission *in vivo* and what the shared underlying mechanisms are between malignant transformation and iPSC induction.

Poster Board Number: T-3151

REPROGRAMMING FACTOR SELECTION USING THE CORE EXPRESSION MODULE ALGORITHM (CEMA)

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The Core Expression Module Algorithm (CEMA) analyzes the transcriptome of a particular cell type versus many other cell types to identify the unique expression patterns of a cell. The CEMA data generates a list ranking the gene expression of the cell of interest. This information can be used to potentially identify transcription factors for reprogramming any cell line. CEMA and data from 10 different cell types constructs a list of the top unique 200 genes that defines each cell line. Evaluation of gene expression levels parsed the gene list down to 3-10 genes that could be valuable for direct reprogramming. Two different polycistronic retroviruses containing candidate transcription factors for keratinocytes are introduced individually into fibroblast for the reprogramming experiment. After the delivery, the fibroblast displayed positive integration of the vector, expressing the venus YFP reporter protein. Immuno-fluorescent staining confirmed the over-expression of the polycistronic genes. Cells will be cultured keratinocyte media for 2-8 weeks and then assayed for reprogramming. Reprogrammed cell will be collected using Fluorescence-Activated Cell sorting (FACs), and expression profiled by RT-PCR and microarray. To functionally assay reprogramming, the calcium switch assay will be employed. Finally, we will test other types of cells for their ability to be reprogrammed. This test will determine if CEMA facilitates the identification of reprogramming factors.

Poster Board Number: T-3152

GENERATION OF NON-INTEGRATING INDUCED PLURIPOTENT STEM CELLS WITH THE CYTOTUNE™ -IPS SENDAI REPROGRAMMING KIT IN CHEMICALLY DEFINED MEDIUM.

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Human induced pluripotent stem cells (iPSCs) are an important potential source of cells for regenerative medicine due to their inherent ability to differentiate into all cell types of the three germ layers. Generation of iPSCs with a non-integrating reprogramming method and in culture conditions that are completely absent of animal proteins will be ideal for such regenerative and cell therapy applications. Here we demonstrate a highly efficient method to generate non-integrating iPSCs using the CytoTuneTM iPS-Sendai Reprogramming Kit. Sendai virus is an RNA virus that replicates only in the cytoplasm and does not integrate into the cellular genome. In addition, we derived non-integrating iPSCs in a chemically-defined medium that does not contain bovine serum albumin. This robust and efficient system of integration-free iPSC generation in defined conditions minimizes the exposure of human cells to animal proteins that may incorporate non-human immunogenic molecules, and will enable human iPSCs for cell therapy-relevant downstream applications.

Poster Board Number: T-3153

MIRNAS ARE REQUIRED FOR INDUCED NEURONAL CONVERSION.

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Our lab has shown that exogenous expression of specific transcription factors can reprogram fibroblast to an induced neuronal cell (iN). Recently, other groups have reported two microRNAs (miRNAs) that enhance iN formation. Because miRNAs are key regulators of biological functions, we wanted to test whether changes in the miRNA population are not only beneficial but also required for the reprogramming process. We have taken advantage of a miRNA deficient mouse embryonic stem cell (mESC) line that lacks DGCR8, a critical component for miRNA processing, to test if miRNAs are required for iN conversion. Wild type (WT) mESCs rapidly convert to Map2+ neurons within 5days upon lentiviral infection with Brn2, Ascl1, and Myt1L (BAM) transcription factors. Meanwhile, DGCR8 -/- mESCs do not show any change in their state when infected with the BAM factors and ultimately die during the reprogramming process. This observation is also apparent with other transcription factor combinations, indicating that mESC directed differentiation towards iNs requires miRNAs. We have also identified several miRNAs that can enhance the iN conversion in WT mESCs when exogenously expressed along with the BAM factors. We are currently screening miRNAs in the DGCR8-/- mESCs to test whether we can rescue the iN conversion deficiency and ultimately use these miRNAs to increase the iN conversion rate.

Poster Board Number: T-3154

PROANGIOGENIC FATE OF BONE MARROW DERIVED STEM PROGENITOR CELLS DURING TISSUE REGENERATION IS DIRECTED BY HOXA3 EXPRESSION

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Bone marrow contains a population of multipotent cells with the capability to proliferate, self-renew, and differentiate into different blood cell types as well as non-haematopoietic partners such as endothelial cells. We and others have shown that during wound repair bone marrow-derived (BMD) stem/progenitor cells migrate to injury site and once there support angiogenesis via providing stimulatory signals such as chemokines and growth factors or directly through differentiation into proangiogenic cells. We have previously shown that soon after injury Hoxa3 is upregulated in many cell types such as keratinocytes, endothelial cells, and BMD progenitor cells, leading to enhanced angiogenesis. Here, we show how Hoxa3 influences the angiogenic potential of BMD haematopoietic stem/progenitor cells as well as mesenchymal stem cells (MSCs). Hoxa3

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promotes the differentiation fate of haematopoietic progenitor cells toward pro-angiogenic Gr-1+CD11b+ cells. In acute wounds, these Gr-1+CD11b+ myelocytic cells promote angiogenesis; however their function is defective under diabetic conditions. In diabetic animals these cells are unable to migrate normally and have a prolonged retention period in the wound milieu. Their gene expression profile is impaired, they do not have normal proliferative capacity, and are more pro-inflammatory. Hoxa3 transcription factor reprogramming of these cells reverts most of these defects and promotes a more pro-angiogenic phenotype, as their gene expression profile shows. Hoxa3 transcription factor reprogramming also enhances the angiogenic potential of MSCs through increasing their migration towards angiogenic signals such as VEGF and enhancing their tube-like network formation capability during their differentiation into endothelial cells. Altogether this suggests Hoxa3 can reprogramme BMD cells into endothelial progenitor-like cells.

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WHOLE-GENOME IDENTIFICATION OF TARGET TRANSCRIPTS AND MOLECULAR PROCESSES MODULATED BY PLURIPOTENCY-RELATED MICRORNAS: MIR-106A AND MIR-302B

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Increasing evidences have accumulated showing that pluripotency-related microRNAs (miRs) can enhance the efficiency of somatic cells reprogramming into induced Pluripotent Stem cells (iPS), when used in association with classic reprogramming factors. Strikingly, transfection of synthetic miRs was shown to directly reprogram somatic cells into iPS cells, opening the possibility of reprogramming without potentially harmful genetic modifications. Nevertheless, the efficiency of synthetic miR-mediated reprogramming is still low, and the molecular mechanisms contributing to the process of reprogramming are only starting to be uncovered. With that in mind, in the present work, we explored the potential roles of the miRs 106a and 302b, centrally involved in miR-mediated reprogramming. For this, synthetic pre-miR, inhibitory anti-miR and corresponding unspecific control molecules were independently transfected into human BJ fibroblasts and into pluripotent NTera2 cells. After 72 hours, whole-genome transcriptomes were obtained by oligonucleotide microarrays. To identify highly confident targets, transcripts downregulated by pre-miR transfection and upregulated by the corresponding anti-miR, in both cell lines, were further compared to the set of predicted targets showing evolutionary conserved miR binding sites (microrna.org). Molecular changes related to the reprogramming process were identified by comparing the microRNAs-induced transcriptional changes, with those observed upon iPS reprogramming. For this, transcriptomes from two iPS and the fibroblasts of origin were used: a partially reprogrammed cell line generated by our group (lentiviral insertion of SOX2, C-MYC and TCL-1A), and that reported by Takahashi (retroviral insertion of OSKM factors). Pathways and biological processes modulated by the miRs were identified using a Functional Annotation Tool (DAVID). Among pathways with a statistically significant enriched number of transcripts, identified as highly confident miR-302b targets, we found: Apoptosis, p53 and WNT signaling (including: MDM4, CDKN1A, CYCS, ATM; IL1R1, LRP6 and ROCK2). Similarly, for miR-106a, the following pathways were identified: Regulation of Actin Cytoskeleton, Adherens Junction, Focal Adhesion, Axon Guidance and MAPK Signaling (including: FN1, FGFR2, LIMK2, PAK2, ROCK1, ROCK1P1, ITGB1, CTNNA1, EP300, TGFBR2, ACVR1B, SMAD2, TAOK1, DUSP3, MAP3K2, PRKCA and PPP3CA). Interestingly, analysis based on shared predicted-targets downregulated by miRs 302b and 106a, in both cell lines, and downregulated upon iPS reprogramming, revealed enrichment of central pathway components of TGFbeta (TGFBR2, SMAD2, SMAD3 and ROCK2) an MAPK signaling (MAPK1, DUSP3, MAP3K2, PAK2). Additionally, although not shared predicted-targets, IL1R1, LTBP2, ROCK1 and GSK3B were similarly modulated. Finally, several transcripts related to pluripotency and reprograming (including Nanog, LIN28B and Nodal) were up-regulated by both miRs and confirmed by qRT-PCR. Our results demonstrate that several components of pathways previously implicated in opposing pluripotency, self-renewal and somatic cell reprogramming, are highly likely directly targeted to degradation by miR-106a and miR-302b. Our findings add to the understanding of the molecular mechanisms by which miRs drive reprogramming, and may help in the future development of miR-mediated reprogramming with increased efficiencies. Support: FAPESP, CNPq and FINEP.

Poster Board Number: T-3157

A SYSTEMATIC APPROACH TO IDENTIFY REGULATORS OF COMPLETE REPROGRAMMING

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Temporal gene expression profiling studies of reprogramming mouse embryonic fibroblast cells that were generated using the doxycycline-inducible *piggyBac* system revealed that reprogramming is a multi-step process that is characterized by Initiation, Maturation and Stabilization phases. Previously, we hypothesized these phases follow each other in a sequential manner and assigned specific markers to each phase of reprogramming. Here we demonstrate that complete Maturation is required for successful transition to Stabilization phase of reprogramming. Using BeadArray and RNA-Seq technologies, we performed clonal transcriptome analysis in a temporal manner during the time course of reprogramming. By comparing gene expression profile of clones that completed Maturation and transited to Stabilization phase to clones that did not, we discovered a gene expression signature associated with successful reprogramming. To determine whether gain of the identified signature is required for transition to Stabilization phase and completion of reprogramming, we established conditions for a systematic RNAi screen to evaluate the function of the candidate genes. In this assay, we took advantage of *piggyBac* secondary reprogramming model and assessed the effect of specific gene knockdown on complete cellular reprogramming. I will present the integrative approach we employed to highlight the significance of some key signaling pathways required for completion of somatic cell reprogramming. Furthermore, the approach and the result of the RNAi screen as well as follow up studies on potential regulators of complete reprogramming will be presented.

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Poster Board Number: T-3158

RECRUITMENT OF THE TRANSCRIPTION MACHINERY FACILITATES REPROGRAMMING

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In order to generate induced pluripotent stem cells (iPSCs), Oct4, Sox2, Klf4 and cMyc need to compete with the endogenous transcription factors for recruiting the transcriptional machinery. Oct4 is considered to be a key player in the pluripotency network and it is essential for the iPSCs generation. We hypothesize that facilitating the access of Oct4 to the transcriptional machinery could increase the reprogramming efficiency. As it has been reported that the adenoviral protein E1A is able to link Oct4 to the basal transcription initiation complex, we decided to include E1A in the reprogramming cocktail. Our data show that E1A increases the iPSC colony formation in neural stem cells and in mouse embryonic fibroblasts. In summary, our results suggest that the recruitment of the transcriptional machinery is a limiting step during the reprogramming process.

Poster Board Number: T-3159

C/EBPA INDUCES B CELL TO MACROPHAGE TRANSDIFFERENTIATION BY ESTABLISHING A STABLE MYELOID TRANSCRIPTION FACTOR NETWORK

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C/EBPa induces B cell to macrophage transdifferentiation by establishing a stable myeloid transcription factor network
C/EBPalpha is a transcription factor capable of inducing the transdifferentiation of committed B cell precursors into macrophages at 100% efficiency. Using an inducible pre-B cell line carrying C/EBPaER we have explored the mechanism by which the myeloid phenotype is established. This line becomes stably committed to a macrophage fate after 24 hours of C/EBPa activation followed by inducer washout. Gene expression profiling over time, combined with gain and loss of function experiments, showed that C/EBPb and PU.1 are required to establish the macrophage state and that endogenous C/EBPa is required to maintain this phenotype. ChIPseq experiments showed that C/EBPa and C/EBPb bind to promoter-distant sites of upregulated genes within 3 hours after induction, and that the transcription factors remain bound to these sites at all later time points. About a third of the sites are already bookmarked by PU.1 in pre-B cells, another third shows a recruitment of PU.1 and the last third remains free of PU.1 binding. The upregulation kinetics of genes in the vicinity of these putative enhancers is significantly influenced by their transcription factor occupancy, as is also reflected by the presence or absence of histone marks that correlate with gene activation (H3K27Ac). Interestingly, the genes closest to the enhancers bookmarked with PU.1 are poised as they are already expressed in pre-B cells at basal levels and then become further activated during transdifferentiation. In contrast, genes corresponding to the two categories of unmarked enhancers are silent in pre-B cells and after C/EBPa binding become de-repressed. In conclusion, C/EBPa induces a stable switch from pre-B cells into macrophages by activating its partners C/EBPb and PU.1. Together the three factors form a stable transcription factor network that first establishes and then maintains the macrophage state.

Poster Board Number: T-3160

MOLECULAR MECHANISMS OF ASTROGLIA-TO-NEURON CONVERSION BY FORCED EXPRESSION OF NEUROG2

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Direct conversion of differentiated cells into neurons is emerging as a promising alternative for cell-based therapies of neurodegenerative diseases. Compared to induced pluripotency, direct conversion is relatively efficient, faster, does not give rise to pluripotent intermediates, and allows for the generation of different types of neurons, depending on the combinations of factors employed. We have focused our attention on the reprogramming potential of glial cells because they are lineage-related to neurons through common radial glia ancestors and are distributed throughout the mammalian brain, thus representing an endogenous source of potential reprogrammable cells. Indeed, we could recently show that retrovirus-mediated expression a single proneural gene - Neurog2 or Mash1 [Ascl1] - can directly and efficiently convert astroglia of postnatal mouse cerebral cortex into fully functional neurons *in vitro*. In order to understand the molecular mechanisms underlying the transgene-mediated conversion of astroglia into neurons, we generated an inducible system in which the Neurog2 cDNA is fused to a modified estrogen receptor-binding domain (ERT2). Therefore, the resultant protein is expressed but it remains inactive unless Ngn2ERT2-expressing cells are treated with tamoxifen, upon which inhibition is released. This allows for a temporally defined onset of the proneural gene's transcriptional activity. Indeed, Neurog2ERT2-transduced astrocytes generated neurons upon tamoxifen treatment, while no neurons were observed in control-treated Ngn2ERT2-transduced cells, thus providing an experimental system for further molecular analyses. To gain insight into the early events of Neurog2-mediated reprogramming, we collected Neurog2ERT2-transduced astrocytes at various time points after tamoxifen treatment - 4, 24 and 48 hours - and performed a microarray-based expression analysis. Interestingly, more than 100 genes were significantly upregulated already 4 hours after tamoxifen application: amongst them, some neurogenic transcription factors are maintained at later stages after induction, while others are only transiently upregulated. Interestingly, none of these genes has a neurogenic effect on astroglia on its own, but they enhance Neurog2-mediated conversion efficiency, and rescue the neurogenic conversion in a model of failed reprogramming. Experiments are ongoing to assess whether these factors are essential for Neurog2-mediated reprogramming, and whether, when expressed altogether, they are sufficient to induce direct neuronal reprogramming in absence of Neurog2.

Poster Board Number: T-3161

DIRECT CONVERSION OF FIBROBLASTS INTO NEURAL STEM CELLS BY DEFINED FACTORS

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Recent advances in stem cell biology have revealed that cell type-specific transcription factors could reset the somatic memory of differentiated cells via direct reprogramming into cells of specific

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identities, such as neurons, cardiomyocytes, blood progenitor cells, hepatocytes, and epiblast stem cells, i.e., without the cells having to pass through an induced pluripotent stem cell state. However, the direct reprogramming of differentiated cells into self-renewing somatic stem cell types has not yet been described. Here we show that a combination of transcription factors can induce mouse fibroblasts to acquire a neural stem cell (NSC) identity. The induced neural stem cells (iNSCs) showed morphology, gene expression, epigenetic features, differentiation potential, self-renewing capacity, as well as in vitro and *in vivo* functionality similar those to wild-type NSCs. Therefore, our data suggest that specific defined factors can induce the direct reprogramming of differentiated somatic cells into specific somatic stem cell identities.

Poster Board Number: T-3162

NUCLEAR WAVE1 IS NECESSARY FOR TRANSCRIPTIONAL REPROGRAMMING BY XENOPUS EGGS AND OOCYTES

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Nuclear reprogramming of differentiated cells holds great promise for regenerative medicine. Reprogramming of differentiated cells can be very efficiently achieved by nuclear transfer to eggs and oocytes. Eggs/oocytes utilize natural reprogramming activity that is used for sperm nuclei to ensure normal embryonic development. However, such egg/oocyte factors and mechanisms involved in reprogramming are not well understood. We have previously shown that nuclear actin which naturally polymerizes in the germinal vesicle (GV), the *Xenopus* oocyte nucleus, plays a key role in reprogramming. Actin polymerization is regulated by actin-binding proteins. We therefore screened actin-binding proteins in the GV as candidate reprogramming factors. We find that WAVE1, which is predominantly expressed in the brain in an adult body, is present in the oocyte nucleus and required for efficient transcriptional reprogramming from mouse nuclei transplanted into the GV. WAVE1 transcript variants are expressed in oocytes and one variant containing the WHD-Basic domain is sufficient to bind to active RNA polymerase II. Interestingly, interaction between nuclear WAVE1 and RNA polymerase II is observed in embryos at the time of zygotic genome activation, at which major transcriptional reprogramming is induced in development, and moreover WAVE1 is important for embryonic development. These results unravel an unexpected role of an actin-binding protein, WAVE1, in reprogramming. This is also a first clear demonstration that a reprogramming factor stored in the oocyte nucleus is used for reprogramming both somatic nuclei and fertilized embryos.

Poster Board Number: T-3163

STRATEGIES FOR IMPROVING DOPAMINE NEURONS DERIVATION WITH DEFINED FACTORS

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Parkinson's Disease (PD) is a neurodegenerative disease characterized by a loss of movement control due to the degeneration of midbrain dopaminergic (DA) neurons in the substantia nigra pars compacta, the brain region that controls voluntary movements. PD predominantly affects elderly people and with an increasing aging world population, it is critical to improve current treatments for this disease. Previous studies utilizing DA neurons derived from ES, iPS and MEF cells have shown various degrees of improving Parkinsonian behavior after transplantation into rodent models. Using Ascl1 (Mash1), Nurr1 and Lmx1a, we report a ~8.6% efficiency of obtaining TH+ cells from MEFs cells. These cells, however, are not positive for another marker PITX3 which is considered a unique defining marker of true midbrain DA cells. A screen of more DA transcription factors yielded candidates that express both TH and PITX3, but the number of double positive cells were few. Current work is focusing on improving the efficiency of obtaining TH+/PITX3+ cells and the performance of these cells *in vivo*.

Poster Board Number: T-3164

THE ROLE OF INDUCED PLURIPOTENT STEM CELLS IN CONSERVATION OF ENDANGERED SPECIES

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*Abstract:*Induced pluripotent stem cells (iPSCs) are generated from somatic cells by direct molecular reprogramming and are capable of unlimited expansion and differentiation into a wide variety of cell types. Mouse iPSCs have been shown to be capable of generating all fetal cell types. These results suggested to us that iPSCs could be valuable tools in the conservation of endangered species. We recently reported on the successful application of reprogramming technology toward the generation of iPSCs from primary fibroblast cultures banked in the Frozen Zoo at the San Diego Zoo's Institute for Conservation Research. We made iPSCs from two critically endangered species, the drill, *Mandrillus leucophaeus*, and the northern white rhinoceros, *Ceratotherium simum cottoni* (Ben-Nun et al., 2011). The Frozen Zoo is an extensive collection of cryopreserved primary fibroblast cultures, collected from over 8600 individual vertebrates from approximately 800 species. During these reprogramming efforts, we determined that Moloney murine leukemia virus-based retroviral vectors could effectively deliver the reprogramming factors into the northern white rhinoceros fibroblasts when pseudotyped with vesicular stomatitis virus G envelope protein (VSV-G), but not with the amphotrophic envelope protein. The VSV-G pseudotyped retroviral vectors also efficiently transduced the drill fibroblasts. We were able to reprogram both the rhinoceros and drill fibroblasts using the human OCT4/POU5F1, SOX2, KLF4, and MYC cDNA sequences, suggesting that retroviral vectors carrying human reprogramming factor sequences and pseudotyped with VSV-G may be widely applicable for generating iPSCs from a variety of species. We are currently using the same methods to reprogram fibroblasts from the Javan Banteng and Somali Wild Ass. The Javan Banteng, *Bos javanicus,* is a species of wild cattle native to Asia. Due to hunting and habitat destruction, the population of wild Javan bantengs has dwindled and they are currently listed as an endangered species[1 1](Timmins, 2008)[1]. The Somali wild ass, *Equus africanus somalicus, is critically endangered with fewer than 200 individuals living in the wild (IUCN Red List of Threatened Species (Moehlman, 2008)). In 2003, it was demonstrated in a collaboration between the* San Diego Zoo's Institute for Conservation Research and Advanced Cell Technology *that somatic cell nuclear transfer of the nucleus from a Javan Banteng fibroblast into an oocyte from a domestic cow could be used to generate a viable animal, using a domestic cow as a surrogate. These results suggest that we may be able to use germ cells, embryos, or surrogates from domestic animals to explore the developmental capabilities of iPSCs from endangered animals.*

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Tissue Engineering

Poster Board Number: T-3165

EFFECT OF PURIFICATION ALGINATE SPONGES WITH CHONDROCYTES FOR THE ARTICULAR CARTILAGE TISSUE ENGINEERING

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Abstract: Adult articular cartilage tissue has poor capability of self-repair. Therefore, a variety of tissue engineering approaches are motivated by the clinical need for articular repair. Non-Purify alginate and purify alginate were fabricated by seed cartilage cell on the sponge. After 1, 5, 7, 10 and 14 day of cell seeding, cell proliferation activity was measured via MTT assay. Morphological observation, histology, biological assay for collagen type I, II and aggrecan, and PCR were performed at each time point 1, 2 and 3 weeks. In result, the cell viability was better in purify alginate sponge than non-purify alginate sponge. Scaning electro microscope (SEM) results showed that more attach cell in the purify alginate sponge with lapse of time. DMMB results showed that the highest content of glycosaminoglycan (GAGs) and collagen at purify alginate sponge. Compression results indicated that purify alginate sponge was the highest intensity. This study suggested that purify alginate sponge may serve as a potential cell delivery vehicle and a structural basis for tissue engineered articular cartilage. This research was supported by WCU (R31-20029). Keywords : Purification Alginate, sponge, chondrocyte WCU BIN Fusion Tech. Secretariat Dept. of BIN Fusion Tech., WCU, Eng. 8th Bldg. rm. No.408, Chonbuk National University, 664-14, Dukjin, Jeonju, 561-756 Korea Tel: +82 63 270 4434 begin_of_the_skype_highlighting +82 63 270 4434 end_of_the_skype_highlighting Fax: +82 63 270 4254 E-mail: bkwcu@jbnu.ac.kr

Poster Board Number: T-3166

VARIATION RATIO OF DBP/ALGINATE MICROCAPSULES WITH CHONDROCYTES FOR THE ARTICULAR CARTILAGE TISSUE ENGINEERING

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Abstract: Adult articular cartilage tissue has poor capability of self-repair. Therefore, a variety of tissue engineering approaches are motivated by the clinical need for articular repair. 0, 0.1, 0.3, 0.5 and 1% DBP/Alginate microcapsules were fabricated by seed cartilage cell into microcapsules. After 1, 5, 7, 10 and 14 days of cell seeding, cell proliferation activity was measured via MTT assay. Morphological observation, histology, biological assay for collagen type I, II and aggrecan, and PCR were performed at each time point of 1, 2 and 3 weeks. In the result, cell viability was higher 1% DBP/alginate microcapsules than the other DBP/alginate microcapsules. DMMB results shown that the highest content of glycosaminoglycan (GAGs) and collagen at 1% DBP/alginate microcapsules. Compression results shown that 1% DBP/alginate microcapsules was the highest intensity. This study suggests that 1% DBP/alginate microcapsules may serve as a potential cell delivery vehicle and a structural basis for tissue engineered articular cartilage. This research was supported by WCU (R31-20029). Keywords : Alginate, Demineralized Bone Powder(DBP), chondrocyte WCU BIN Fusion Tech. Secretariat Dept. of BIN Fusion Tech., WCU, Eng. 8th Bldg. rm. No.408, Chonbuk National University, 664-14, Dukjin, Jeonju, 561-756 Korea Tel: +82 63 270 4434 begin_of_the_skype_highlighting +82 63 270 4434 end_of_the_skype_highlighting Fax: +82 63 270 4254 E-mail: bkwcu@jbnu.ac.kr

Poster Board Number: T-3167

STRUCTURE AND PROPERTIES OF INJECTABLE GELLAN GUM HYDROGEL

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Abstract: Gellan gum (GG) is an exopolysaccharide produced by Sphingomonas elodea. In this study we prepared three dimensional hydrogel of GG reacted with 1-ethyl-3-(3-di-methylaminopropyl) carbodiimide (EDC) to obtain a cross-linked GG. From the FT-IR analy- sis, the mechanism of EDC cross-linking in the GG was the condensation reaction between the carboxyl and hydroxyl group. Because of polysaccharide hydrogels normally possess poor mechanical strength, the results obtained in this study indicated that GG was sutiable for potential applications in tissue regeneration. In vitro biocompativility tests, hydrogel ex- hibited nontoxic effects. When implanted in to rat subcutaneous tissue, implantation showed that hydrogel caused slight inflammation in the first few days after operation. In the tissue engineering, cross-linked GG demonstrated good physical properties and biocompatibility, and has great potential for future use. This research was supported by WCU (R31-20029) and MBC (0405-BO01-0204-0006). Keywords: Gellan gum, Hydrogel, Biocompativility WCU BIN Fusion Tech. Secretariat Dept. of BIN Fusion Tech., WCU, Eng. 8th Bldg. rm. No.408, Chonbuk National University, 664-14, Dukjin, Jeonju, 561-756 Korea Tel: +82 63 270 4434 begin_of_the_skype_highlighting +82 63 270 4434 end_of_the_skype_highlighting Fax: +82 63 270 4254 E-mail: bkwcu@jbnu.ac.kr

Poster Board Number: T-3168

EFFECT OF GELLAN GUM HYDROGEL FOR CARTILAGE REGENERATION

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Abstract: Injectable hydrogels have been studied for potential applications for articular cartilage regeneration. In this study, our aim is to research effect of gellan gum hydrogel for cartilage regeneration on the cell viability and extracellular matix (ECM) secretion. The preparation of gellan gum hydrogel was designed by varying the contents with 1, 2 and 3 wt%. Hydrogels using 1, 2 and 3 wt% of gellan gum were fabricated with cross-linking by EDC (1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide). Cell viability (MTT), glycolsamino-glycan (GAG) assay, histological examination were evaluated to analyze the effect of the gellan gum hydrogels on the cell viability and ECM secretion. The gellan gum hydrogels cross-linked by EDC became more increase the rate of cell growth than non cross-linked gellan gum hydrogels. Also the phenotype of the cells maintained better in gellan gum hydrogels having 3 wt% gellan gum contents than the others. These results demonstrated that the gellan gum would significantly affect the cell metabolism. This research was supported by WCU (R31-20029) and MBC (0405-BO010204-0006). Keywords: Gellan gum, Cartilage, disc, EDC(1-ethyl-3-(3-dimethyl aminopropyl) carbodii mide)

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Poster Board Number: T-3169

THE INFLAMMATORY RESPONSES TO PLGA/DBP/SIS/SILK SCAFFOLDS IN VITRO AND *IN VIVO*

Lee, Yu Jeong, Jang, Ji Eun, Jeon, Dae Yeon, Lee, Dongwon, Khang, Gilson

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Poly (lactide-co-glycolic acid) (PLGA) has been widely applied to tissue engineering as a good biocompatible materials. However, there degradation products can decrease the pH in the surrounding tissue, which can result in inflammatory reaction. We hypothesized that the PLGA implant might have an inflammatory responses that could be reduced by impregnating demineralized bone particles (DBPs), porcine small intestinal submucosa (SIS) and silk into the PLGA. We manufactured three different materials of PLGA scaffolds, with each materials containing 20 wt% of DBPs, SIS and silk of PLGA. For biocompatibility test, NIH/3T3 mouse fibroblasts were cultured in the PLGA/DBP/SIS/Silk scaffold for 3days. The PLGA/DBP/SIS/Silk scaffolds had no adverse effect on NIH/3T3 cell attachment and did not affect cell viability. The inflammatory potential of PLGA was evaluated using messenger ribonucleic acid expression of tumor necrosis factor alpha (TNF-α) and reactive oxygen species (ROS) on a mouse leukaemic monocyte macrophage cell line (RAW264.7). This study showed that DBP/SIS/Silk scaffolds reduction of inflammatory reaction of PLGA.This research was supported by WCU (R31-20029) and MBC (0405-B001-0204-0006).

Poster Board Number: T-3170

THE EFFECTS OF HESPERIDIN ON THE INFLAMMATORY RESPONSE; IN VITRO AND *IN VIVO*

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Hesperidin, a flavonoid present in fruits and vegetables, possesses anti- inflammatory and chemopreventive effects. In this work, hesperidin-incorporated Poly (lactide-co-glycolic acid) (PLGA) films were manufactured to evaluate the effects of hesperidin on the inflammatory responses. *In vitro* inflammatory responses to murine macrophage RAW264.7cells were cultured on the PLGA/hesperidin film for 1, 3 and 5 days. Hesperidin films minimally stimulated the cells to generate less production of tumor necrosis factor alpha (TNF-α) and reactive oxygen species (ROS) then PLGA films. The *in vivo* responses to PLGA/hesperidin films implanted in the axilla skin of rat. Fibrous wall thickness and macrophage were evaluated by hematoxylin and eosin (H&E) and ED-1 immunohistochemical staining, respectively. Based on the low level of inflammatory responses, hesperidin has great potential for biomedical applications. This research was supported by WCU (R31-20029) and MBC (0405-B001-0204-0006).

Poster Board Number: T-3171

PROLIFERATION AND DIFFERENTIATION OF SCHWANN CELL IN SIS SPONGE OF VARIOUS CONTENTS

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The porcine small intestinal submucosa (SIS)consists of some growth factors which can stimulate cell activity without immune rejection responses. Thus, SIS had been widely used as a biomaterial. In this study, we evaluated the effect of content SIS sponge on proliferation and differentiation of schwann cells (SC). SCs were harvested from the femurs and tibias of female Fischer rat. SIS sponge were prepared by freeze-drying method from 1, 2 and 3 wt% SIS solution. The SCs were seeded in SIS sponge with 2×10^5 cell/sponge concentration. The effect of SIS sponge on the proliferation and differentiation of SCs were assessed in culture using the MTT assay, SEM, swelling test. RT-PCR was conducted to confirm mRNA expression of S100, NSE, p75 and NF for nerve marker. In addition, immunochemical evaluation for cellular proliferation and differentiation by H&E and anti-NF staining. Theses studies revealed that SIS sponge has a positive effects on the SCs growth and the phenotype maintain through the study. This research was supported by WCU (R31-20029) and SCRC (SC4110).

Poster Board Number: T-3172

SPINAL CORD REGENERATION USING BONE MARROW STROMAL CELL WITH PLGA/DBP NERVE CHANNEL CONTAINED ALGINATE

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Poly (L-lactide-co-glycolide) (PLGA) is a biodegradable synthetic polymer with acceptable mechanical strength and well-controlled degradation rate. Also, it can be easily fabricated into many shape. Demineralized bone particle (DBP) that affects to cell proliferation and differentiation has been used as biomaterial. Bone marrow stromal cells (BMSCs) exhibit multiple traits of a stem cell population. We proposed to use PLGA nerve channel impregnated with DBP and Alginate by tissue-engineering principles for the repair of spinal cord injury. The spinal cord was completely transected horizontally at two levels (T7 and T8) and PLGA/DBP nerve channel seeded the cells were implanted in the lesion. For histological and immunochemical evaluation, the implants were removed after 2, 4 and 8 weeks and H&E and anti-NF staining. Motor functional outcome measurements using the BBB scoring, sensory test and motor functional recovery test were performed every week for 8 weeks post injury. This study suggested that BMSCs and Alginate contained to PLGA/DBP nerve channel may have an important role for spinal cord regeneration of tissue engineering area.
This research was supported by WCU (R31-20029) and SCRC (SC4110).

Poster Board Number: T-3173

TISSUE ENGINEERING TECHNOLOGY INDISPENSABLE FOR CELL-BASED REGENERATIVE MEDICINE

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A new therapeutic trial based on the natural healing potential of body itself to induce tissues regeneration and repairing, has been recently expected. The natural healing is based on the potential of cells for their proliferation and differentiation. Now several stem cells with high potentials have been available through the recent development of stem cell biology. However, only when the cells are transplanted without considering any their local environment, we cannot always expect the cell-based tissue regeneration. This is because in the body, generally cells survive and biologically function by interacting with their local environment. The environ-

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ment is composed of signaling molecules and extracellular matrix of natural scaffold which correspond to the food and house of cells, respectively. Tissue engineering is a biomaterial technology or methodology to artificially create the local environment of cells which can assist their inherent potential for tissue regeneration. If a cell scaffold or a key signaling molecule is supplied to the right place at the right time period or concentration, the body system initiates to physiologically function, resulting in the natural induction of cell-based tissue regeneration. The biological functions of signaling molecules with *in vivo* in-stability can be augmented with drug delivery system (DDS) technology. For example, biodegradable hydrogels are designed for the controlled release of biologically active signaling molecules to experimentally and clinically succeed in the cell-induced regeneration and repairing of various tissues. This DDS and/or cell scaffold technologies can be combined with cell transplantation to significantly enhance the therapeutic efficacy in tissue regeneration. The injection of hydrogel for basic fibroblast growth factor (bFGF) release induced angiogenesis at the injected site. When stem cells were transplanted at the angiogenesis-induced site, their biological functions were promoted to demonstrate enhanced therapeutic efficacy, in contrast to those without angiogenic induction. In addition, the dual release of two signaling molecules can be achieved by the hydrogel. When both stromal derived factor (SDF)-1 and bone morphogenic protein (BMP)-2 were released in the subcutaneous tissue, ectopic bone formation was observed to a significantly great extent compared with that of either release. The SDF-1 release enhanced the recruitment of cells from the bone marrow, while the osteogenic differentiation of cells recruited was promoted by the BMP-2 release. The DDS technology can manipulate the body distribution and fate of cells, which enhances the natural healing potential for cell-induced tissue regeneration. The biomaterials technology of regenerative medicine is also applicable to the basic researches of stem cells biology. The further development of stem cell biology will accumulate the scientific knowledge of regenerative medicine, resulting in enhancing the therapeutic efficacy of cell-based tissue regeneration. In this paper, several applications of DDS and cell scaffold technologies to the tissue regeneration therapy as well as the basic research of stem cell biology are introduced to emphasize significance of tissue engineering technologies in regenerative medicine.

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LIVER ANTI-FIBROSIS THERAPY WITH MESENCHYMAL STEM CELLS GENETICALLY ENGINEERED BY SPERMINE-INTRODUCED PULLULAN

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Liver fibrosis is one of the intractable diseases. Now a clinically possible therapy for liver fibrosis is liver transplantation. However, the lack of donor liver is the large problem. Another therapeutic choice is cell transplantation. Mesenchymal stem cells (MSC) can be readily isolated from patients and possess the potential of good proliferation. It is reported that MSC have an inherent ability to naturally recruit to an injury tissue. In addition, MSC have some characteristics suitable for cell therapy. They can differentiate into different lineages of cells, such as osteocytes, chondrocytes, adipocytes, myocytes, epithelial cells, and neurons, while their differentiation into cardiocytes and hepatocytes, is experimentally demonstrated. Another characteristic is to secret bioactive factors, such as tumor necrosis factor, interleukine-6, and hepatocyte growth factor (HGF), which positively affect the biological functions of the surrounding cells. Among them, HGF shows anti-apoptotic, migration, proliferation, and anti-fibrotic functions. However, considering their transplantation for cell therapy, the low viability of cells and the consequent poor functions are of therapeutical problem. As one trial to break through the problems, the cells genetically engineered to enhance their functions are promising. From the clinical viewpoint, the genetically engineering of cells through their transfection with non-viral carriers is preferable. This study is undertaken to design a novel gene carrier of cationic polysaccharide for the receptor-mediated gene transfection. MSC are reported to express an asialo-protein polysaccharide receptor. We prepared a spermine-introduced pullulan of non-viral vector and the therapeutic effect of MSC genetically engineered by spermine-introduced pullulan was investigated. Spermine was chemically introduced to the hydroxyl groups of pullulan by N, N'-carbonyldiimidazole activation method. MSC were genetically engineered by transfection with the complex of the spermine-introduced pullulan and a plasmid DNA of HGF. HGF secretion from MSC transfected was evaluated. The biological activity of HGF secreted was assayed in terms of hepatocytes proliferation. The anti-fibrotic effect of genetically engineered MSC was evaluated for a rat model of liver fibrosis. When MSC were transfected by the complex of HGF plasmid DNA and the spermine-introduced pullulan, the MSC secreted HGF with biological activity for hepatocytes over 1 week. After intravenous infection into rats with liver fibrosis, the HGF-secreting MSC accumulated in the liver and decreased the fibrosis area to a significantly great extent compared with the original, non-transfected MSC. It is concluded that the transplantation of genetically-engineered MSC is a promising therapy for liver fibrosis.

Poster Board Number: T-3175

ELECTROSPRAYING TECHNIQUE EFFECTS ON HUMAN MESENCHYMAL STEM CELLS

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Electrospinning (ES) is a method widely used in bioengineering to produce nanofibrous scaffolds that mimic the extracellular matrix. These scaffolds act as supports for growing stem cells (SCs) and this combination offers a promising tool for the recovery of organs and tissues. Normally, the scaffolds are produced by ES and subsequently the cells are seeded onto their surface for a tridimensional culture. Alternatively, by combining ES and electrospraying (EP) methods, SCs can be incorporated into the tridimensional scaffolds while the nanofibers are being produced. EP is a technique that can be used for processing SCs in suspension, which is subjected to an electric field. The suspension fluid is then passed through a thin needle and is fragmented, generating droplets containing cells. This ensures that the cells are evenly distributed throughout the scaffold structure to form a 3D system. In this study, the effects of EP method on mesenchymal stem cells (MSCs) from deciduous teeth were evaluated in terms of viability, proliferation, plasticity and genotoxicity. The MSCs, in the sixth passage, were suspended in culture media at $3x10^6$ cells/mL and electrosprayed into a petri dish containing HDMEM media. The parameters: 15 kV, 4 cm and

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0.52 mL/h of EP were chosen to generate a continuous, directed and stable stream of cells. MSCs that were not electrosprayed were used as the control group for all experiments. The assessment with trypan blue revealed no significant loss in viability of MSCs, with a viability of not less than 87%. The MSCs were submitted to EP at different times (15 - 60 min), plated (5,000 cells/cm^2) and cultured at 37°C in 5% CO_2 atmosphere. The electrosprayed cells were able to adhere to culture flasks in the same time as the control group and did not exhibit any form of cellular damage. The MTT test showed no changes in the proliferation of cells and, like the control group, proved to be confluent in 15 days of culture. To examine the maintenance of plasticity, the cells were evaluated in terms of differentiation potential. The electrosprayed MSCs were able to differentiate into chondroblasts, osteoblasts and adipocytes in the same time as the control cells. To assess possible DNA damage after the cells were processed by EP at different times, comet assay was performed. The electrosprayed MSCs were evaluated with comet assay immediately after the EP and after 3 hours of culture. The results indicated no damage to these cells until 15 min of electrospraying. However, the MSCs that were submitted to 60 minutes of EP showed genetic damage ($p<0,01$) when the comet assay was realized immediately after the EP. On the other hand, when these cells were cultivated for 3 h before the comet assay, the genetic damage was not observed, suggesting that cells are able to repair the DNA. This hypothesis is corroborated by the fact that the cells submitted to EP maintain cell viability, plasticity and proliferation in a similar way to the controls. Karyotyping of electrosprayed cells is being performed to evaluate chromosomal abnormalities. These results suggest that EP can be safely used as a technique for incorporating stem cells on scaffolds produced by ES, making it a viable alternative for use in tissue engineering.

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HUMAN MESENCHYMAL STEM CELLS CULTIVATED ON EPIDERMAL GROWTH FACTOR LOADED SCAFFOLDS: A BASIS FOR TISSUE ENGINEERING

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A strategy to optimize tissue regeneration is the use of nanofiber matrices containing growth factors (GFs). This is to ensure correct distribution of stem cells, enhancing their proliferative and differentiation capacity at the injury site, thus preventing the cells from migrating to other locations. The bioavailability of these factors can be obtained by incorporation GFs into nanofibres by electrospinning. The aim of this work was to produce aligned nanofiber matrices with incorporated epidermal growth factor (EGF) and to evaluate the influence of these scaffolds when mesenchymal stem cells (MSCs) are cultivated and differentiated into neural precursors into the scaffolds. The polymer solution consisting of poly(lactic-*co*-glycolic acid) (PLGA) was produced at a concentration of 15% (w/w) using 1,1,1,3,3,3-Hexafluoro-2-propanol with 0.2% of Span 80, forming the oil phase. An aqueous solution of PBS containing 0.1% of albumin and 1mg/mL of EGF was mixed with the oil phase to form the emulsion. Aligned scaffolds with emulsion, without EGF (PLGA, group 1) and with EGF (PLGA/EGF, group 2) were produced using a cylinder rotating at 2,500 rpm. The control group was the cells cultured on wells. The matrices were evaluated for morphology and fiber diameter by scanning electron microscopy (SEM). The MSCs were extracted from human deciduous teeth pulp, called SHEDs (Stem cells from Human Exfoliated Deciduous teeth) and used in the experiments in the 5th passage. The dental pulp was removed and the cells were treated with collagenase at 37°C for 60 minutes, centrifuged, washed and prepared for cell culture. For neuronal differentiation, 5x10^3/cm^2 and 10x10^3/cm^2 cells were seeded in culture plates of 24 wells (control group) and on scaffolds, respectively, and then treated with DMEM supplemented with 30µM of retinoic acid. After 14 days, the differentiation was evaluated by phase contrast microscopy and immunofluorescence using primary antibodies, nestin and GFAP. PLGA and PLGA/EGF scaffolds showed aligned fibers, homogeneous morphology and absence of beads, with an average diameter of 548±29nm and 329±87nm, respectively. By confocal microscopy, it is possible to observe that SHEDs adhere on scaffolds following the same orientation of aligned fibers in group 1 and 2. Concerning neuronal differentiation, samples cultivated on the control group showed cells with neural phenotype, labeling positively for nestin and GFAP markers. The differentiation on group 1 and 2 is still in progress. The small and more variable diameter of the PLGA/EGF (329±87nm) is interesting because it mimics the collagen fibers of the extracellular matrix, where the diameter varies from 50 to 500nm. Through observation of the SEM photographs, it is possible to observe that the scaffolds have a high alignment and porosity. It allows for a three-dimensional arrangement of the cells in the scaffold and permits cellular growth and differentiation. SHEDs are capable of differentiating into neural precursors on the plates and adhere on aligned PLGA/EGF. Despite the neuronal differentiation in scaffolds not being complete, it is speculated that SHEDs will also be able to differentiate under the matrices and increase the expression of nestin and GFAP, due to the stimulus provided by the scaffolds, especially in group 2. Therefore, SHEDs cultivated on aligned PLGA/EGF can be a possible option for tissue engineering to treat neurological diseases.

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ASSOCIATION BETWEEN HUMAN ADIPOSE DERIVED STEM CELLS AND PLGA/VEGF SCAFFOLDS FOR TISSUE ENGINEERING

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Electrospinning (ES) technique permits formation of nanofibres matrices that mimic, in structure and dimensions, collagen fibers of the natural extracellular matrix. The association of bioactive molecules, such as vascular endothelial growth factor (VEGF), in nanofibres allows the controlled release of the encapsulated/incorporated agent. It can contribute to cellular migration, viability and differentiation, becoming an interesting option for tissue regeneration. The aim of this study was to analyze the impact of VEGF incorporated on a poly(lactic-co-glycolic acid) (PLGA) 3D scaffold produced by ES on viability and adhesion of human adipose-derived stem cells (hADSCs). The scaffolds were produced from emulsion as follows: organic phase consisting of PLGA 15% (w/w), sorbitan monooleate (Span-80) 0.2%, hexafluoro-2-propanol and 0.1 % aqueous phase containing PBS, VEGF and BSA. Bovine serum albumin (BSA), acting as carrier protein, stabilizes growth factor during incorporation

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process. The followed physicochemical analyses were carried out: (1) morphology by scanning electron microscopy (SEM), (2) fibre diameter by Image J software, (3) VEGF release by ELISA and (4) VEGF loading analysis by DSC (differential scanning calorimetry). The association between scaffolds and hADSCs was evaluated by: (1) adhesion tests through DAPI fluorescence after 6 hours of incubation, (2) viability by MTT for 1, 4, 7 and 14 days and (3) cell morphology on scaffolds by confocal microscopy. For biological assays three groups were evaluated: (1) PLGA; (2) PLGA/VEGF and (3) control group, in which the cells were cultivated directly on wells. hADSCs were obtained from elective liposuction procedures. The cells were characterized by immunophenotyping profile and differentiated into adipocytes, chondroblasts and osteoblasts. The nanofibres showed a smooth surface without beads for both groups with an average diameter of 604.0±95.80 nm and 626.86±89.94 nm for PLGA/VEGF and only PLGA, respectively. VEGF release from scaffolds reached 54% of the incorporated protein after 14 days. The scaffolds with and without VEGF differ in their exothermic peaks obtained by DSC, suggesting the growth factor incorporation. Biological tests showed that matrices with VEGF had a higher cell adhesion when compared to control group (p=0.007). This suggests an increased bioactivity in scaffolds containing the growth factor. Morphology of the cells seeded on PLGA/VEGF scaffolds during 6h did not differ from the morphology of cells seeded on PLGA scaffolds, confirming that PLGA/VEGF scaffolds can be used for the hADSCs growth. hADSCs showed actin filaments prolongation on both types of scaffolds after 6 hours of cultivation. It indicates that these cells were satisfactorily adapted to scaffolds. In relation to the viability assay, it was observed that the PLGA/VEGF scaffolds have a greater trend toward increased viability rate compared to other groups. It suggests that PLGA/VEGF scaffolds may be a good carrier for VEGF releasing, improving the hADSCs adhesion and viability. Thus, the association between growth factors loaded scaffolds and stem cell is a promising alternative for tissue engineering.

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NGF LOADED POLYMERIC NANOFIBER STANDARIZATION IN ASSOCIATION WITH HUMAN MESENCHYMAL STEM CELLS FOR NERVE TISSUE ENGINEERING

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The use of polymeric matrices or scaffolds composed of nanofibers produced by electrospinning (ES) can serve as templates in which the cells can adhere, proliferate and differentiate, contributing to the development of a new tissue. To optimize the process of tissue regeneration, the cell growth in the scaffolds can be stimulated through the controlled release of bioactive molecules, such as neurotrophic factors. The nerve growth factor (NGF), for example, can be highlighted when associated to nerve tissue engineering models. The main objective of this work is to develop poly (lactic-*co*-glycolic acid) (PLGA) nanofiber matrices loaded with NGF and evaluate the behavior of mesenchymal stem cells (MSCs) on these three-dimensional devices. The MSCs were extracted from human deciduous teeth pulp (hDTP), called SHEDs (Stem cells from Human Exfoliated Deciduous teeth). To permit NGF incorporation, the scaffolds were produced by emulsion, as follows: organic phase consisting of PLGA 15% (w/w), sorbitan monooleate (Span-80) 0.2%, 1,1,1,3,3,3-hexafluoro-2-propanol and 0.1 % aqueous phase containing PBS, NGF and Bovine Serum Albumin. The following scaffolds were developed, characterized and evaluated: (1) NGF loaded aligned scaffolds, (2) NGF loaded random scaffolds, (3) aligned scaffolds without NGF and (4) random scaffolds without NGF. Cultured cells on plate wells were used as the control group. The physicochemical analysis fiber morphology and diameter by scanning electron microscopy (SEM), NGF incorporation assessment by differential scanning calorimetry (DSC) and NGF loading efficiency by ELISA (enzyme-linked immunosorbent assay) were carried out. Cell adhesion on the scaffolds was assessed by DAPI fluorescence (3 and 6 hours of incubation) and cell morphology through confocal microscopy. In all groups, nanofibres with homogeneous morphology without beads were observed. The average diameter was 559±198 nm in group 1, 673±32 nm in group 2, 548±29 nm in group 3 and 754±300 nm in group 4. DSC analysis indicates the presence of NGF in the scaffolds and the average loading efficiency was 2.68±0.83%. The adhesion test showed no significant difference for the tested scaffolds in both times (3 and 6h) compared to the control group. This indicates a satisfactory interaction between the cells and the scaffolds. Cells settled on the scaffolds were observed by confocal microscopy, showing extension of actin filaments. The low efficiency of factor incorporation corroborates with previous results from the literature. NGF release profile from scaffolds, citotoxicity and viability studies will be performed, along with neural differentiation analysis by gene expression of the following markers: nestin, β-III tubulin, Neuron Especific Enolase (NSE), Glial Fibrillary Acidic Protein (GFAP) and Olig 2. These preliminary results suggest appropriate cell adhesion on scaffolds, indicating a good environment for cell growth, which will possibly be enriched by NGF released from the scaffolds. As is well known, growth factors are potent molecules, which even at low concentrations can improve cell behavior and stimulate differentiation. Therefore, using nanotechnology for the incorporation of NGF in the scaffolds, the association of these scaffolds and MSCs is a promising strategy for nerve tissue engineering.

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EVALUATION OF BONE REGENERATION IN RAT CALVARIA USING POLYMERIC NANOFIBER MATRICES SEEDED WITH STEM CELLS FROM HUMAN DENTAL PULP OF DECIDUOUS TEETH

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Bone loss is a challenge for reconstructive surgery, where the use of different sources of bone grafts with physical, chemical and biological limitations is common. Currently, bioengineering combines knowledge from different areas seeking new forms of tissue production, including for use in reconstructive surgery. The aim of this study was to associate nanotechnology with stem cell therapy, to study bone formation in bone defects created in rat calvaria. Scaffolds of acid poly (lactic-co-glycolic acid) (PLGA) were produced by electrospinning technique and characterized for their morphology. Five samples of human stem cells from deciduous teeth (SCDT), in a process of rhizolysis were grown until the 5th passage. They were seeded onto culture plates (control) and scaffolds (test) for adhesion and cell viability assays. To evaluate their ability to promote bone formation, 15 Wistar rats were used, in which critical defects were produced with a 8.0 mm diameter in the calvaria. The animals were divided into three groups (n=5): I- only scaffolds; II- scaffolds/SCDT; III- scaffolds/SCDT, maintained for 13 days in osteogenic medium. Sixty days postoperatively, the calvarias were removed for histometric analysis of the amount of new bone. This study was approved by the Ethics Committee of Federal University of Rio Grande do Sul (n° 19273). Through the images obtained by scanning electron microscopy, it was observed that the fibres formed (mean diameter of 315.9 ± 70.2 nm) were smooth and randomly distributed throughout the scaffold structure, which presented a large number of interconnected pores and a thickness of 37 ± 5.3 µm. Using cell adhesion assay, the same observations were found in the control and test groups, with 44.8 ± 23.1 and 51.6 ± 20 cells per field respectively and with no statistical difference between the groups. Cell viability increased progressively in the beginning of the experiment up to 14 days, followed by a decrease on day 21. Cell viability remained similar between the test and control groups in the different experimental periods with no statistical difference. Histological examination showed that group III, using osteogenic medium, showed 17 ± 4.31% of new bone formation at the site of the original defect, with statistical difference compared to the other groups (9.39 ± 2.55% - group I and 10.7 ± 3.22% - group II). In the light of these results, it is concluded that the PLGA scaffolds produce favorable results in relation to the interaction with the SCDT. The use of PLGA scaffolds and SCDT in association with osteogenic medium, prior to implantation has been shown to have the ability to promote bone formation and ,therefore, it is appropriate for use in bioengineering.

Poster Board Number: T-3180

THREE DIMENSIONAL PROLIFERATION AND MIGRATION OF HUMAN MESENCHYMAL STEM CELLS

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Thus far, human mesenchymal stem cells (hMSCs) have been shown to sense, respond and adapt to various sources of physical, chemical and biological stimuli. Though how these factors converge in three dimensions (3D) to govern cell proliferation and reinforce tissues' homeostasis remains unknown. In this study, we investigated the multidimensionality of hMSCs proliferation in a clinically-relevant size scaffold (70 µl). To achieve strict control of hMSCs cultures of three human donors, spatial (i.e. surface area and hMSCs position) and culture (i.e. cell number/cm^2) parameters were chemically and physically manipulated by surface modification and scaffold fabrication techniques in two dimensions (2D) and 3D. We began by exploring hMSCs proliferation in time, which led to the finding that hMSCs displayed significantly lower specific growth rates in 3D than in 2D. Ostensibly, hMSCs' specific growth rate in 3D is strongly influenced by molecular gradients (e.g. glucose) present in scaffolds which are time-and-space dependent. p21 and p27 cell cycle regulators and cell death assays indicated that despite steep gradients in scaffolds, hMSCs did not die or stop dividing. Furthermore, we assessed nutrient availability through glucose kinetics and unraveled higher glucose consumption rates in 3D than in 2D, corroborating that hMSCs' phenotype in time is dimension-dependent. Additional glucose/cell in 3D suggested a shift in the carbon balance towards the production of extracellular matrix components. Therefore, total collagen and glycosaminoglycans (GAGs) were measured to verify the direction of carbon in glucose molecules. However, neither collagen/cell nor GAGs/cell were higher in 3D. Upon microscopic inspection, it was observed that hMSCs consistently migrated into millimeter-size circular patterns in the scaffold's pores which are structurally sustained by GAGs. Thus, another possibility was that extra glucose/cell in 3D is necessary for organized hMSCs migration, which constitutes an energy demanding process. Interestingly, the circular shape of hMSCs organization in the scaffolds' pores correlated with the shape of concentration gradients of glucose, suggesting a chemotaxis-guided hMSCs migration towards regions of high concentration of molecules. These data implied that nutrient availability has a morphogenetic effect, which prompts hMSCs proliferation and migration. To understand how the molecular gradients in the 3D environment influence hMSCs, we are performing a genome-wide microarray analysis of cultures to connect molecular and physical mechanisms of hMSCs proliferation and migration. These are relevant to understand the homeostasis of tissues and to implement stem-cell-driven tissue and organ regeneration.

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GENERATION OF ISLET LIKE STRUCTURES WITH HUMAN FUNCTIONAL VASCULAR NETWORKS

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Objective" Pancreatic islets are highly vascularized, which is important in their ability to quickly secrete insulin in response to changes in blood glucose. Islet transplantation is an alternative to whole pancreas transplantation in curative therapy of type I diabetics. The outcome of engraftment of islet, however, remains disappointing. Rapid and adequate islet revascularization is crucial for the survival and function of transplanted islets. Here, we describe the induction of functional vascular networks into an islet-like tissue through the vascular-mesenchymal interactions. Methods: Pancreatic beta-cell lines (MIN6) were three-dimensionally (3D) cultivated with endothelial cells and mesenchymal cells. Timelapse confocal microscope analyses were performed to visualize the dynamic cellular organizing process with the use of fluorescence labeled cells. In vitro prevascularized constructs were transplanted under the transparency window of immunodeficient mice. The process of functional vascular network formation was intravitally monitored for over 1 month. Generated constructs were harvested and histologically examined. Results: We successfully integrate the premature vascular network into be-ta cell clusters following the 3D co-cultivation in vitro. After transplantation in immunodeficient mice, generated clusters were permeated with host blood vessels *in vivo*. The patency of human vascular structures was proved by rhodamine dextran infusion. These functional vascular structures were remained for over 2 weeks. Vascular quantification showed the dense vascular networks compared with control samples. Histological analyses revealed the reconstitution of basement membrane proteins, which contributes maintenance of be-ta cell functions. Discussion: One ultimate goal of regenerative medicine is to reconstitute 3D vascular structures with fully functioning mature cells. Vascularization is essential to maintain cell viability during tissue growth, induce structural organization and promote vascularization after implantation. We successfully introduced the vascular networks into the islet-like tissue. Our preliminary results should facilitate future efforts towards the establishment of highly efficient islet transplantation techniques including long-term survival and function of islet grafts.

Poster Board Number: T-3182

ALIGNED VASCULAR GRAFTS USING INDUCED HUMAN PLURIPOTENT STEM CELL-DERIVED ENDOTHELIAL CELLS MODULATE CELL MORPHOLOGY AND VIABILITY

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Cell-based approaches to restore or regenerate a healthy vascular endothelium by enhancing angiogenesis are promising for the treatment of ischemic vascular disease. We have previously shown that human induced pluripotent stem cell-derived endothelial cells (iPSC-ECs) can enhance blood flow and neovessel formation in animal models of peripheral arterial disease. However, in order to replace large conduit vessels, bioequivalent vascular grafts that sustain viability and mimic the longitudinal alignment of native endothelial cells (ECs) are desired. Therefore, the purpose of this study was to examine the effect of aligned nanofibrillar biomaterials on iPSC-EC behavior and survival. We developed a novel method to produce highly organized nanofibrillar collagen scaffolds and characterized their effect on the morphology and survival of iPSC-ECs. Shearing of purified monomeric collagen I solution in the liquid crystal state enables the collagen molecules to self-assemble into parallel-oriented nanofibrils with controllable fibril diameters. Uniform areas of aligned collagen fibrils were fabricated, as confirmed by atomic force microscopy and scanning electron microscopy. Human adult dermal fibroblast-derived iPSCs were differentiated into endothelial lineage in the presence of differentiation media containing BMP4 and VEGF, and then purified based on the expression of a mature phenotypic EC marker, VE-cadherin. The iPSC-ECs were expanded and characterized for EC phenotype by the expression of known EC phenotypic markers, uptake of acetylated-LDL, and the formation of tube-like structures in matrigel. When cultured on 30-nm diameter nanofibrils, the iPSC-ECs became elongated and reorganized their F-actin cytoskeletal assembly along the direction of the fibrils, mimicking the longitudinal orientation of healthy ECs in straight segments of vessels.

Poster Board Number: T-3183

INCREASE OF VASCULARIZATION USING ALLODERM ASSOCIATED WITH DIFFERENT SOURCES OF MESENCHYMAL STEM CELLS FOR PALATAL WOUND IMPLANTATION

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BACKGROUND: Tissue shortage complicates the surgery of cleft lip and palate anomalies. The healing of defects on the palate impairs growth of the dento-alveolar complex due to scar tissue formation. Implantation of substitutes into the wound area might overcome this adverse effect. It is known that palatal wounds treated with a dermal substrate (alloderm) heal with fewer indications of scar tissue formation and evoke only a mild inflammatory reaction. The aim of this study was to compare the tissue response in concern to revascularization properties using AlloDerm associated with mesenchymal stem cells obtained from different sources: dental pulp, levator palatal muscle and adipose tissue to assist in faster tissue integration. This can be helpful to find a good source of stem cell to be associated with AlloDerm for future clinical application in patients with cleft palate to increase the vascularization process. Methods: Adipose-derived stem cells (ASC) were obtained from liposuction patients without clef lip and palate. Dental pulp stem cells (DPSC) and levator palatal muscle-derived stem cell (EPMDSC) were obtained from cleft lip and palate patients. The cells were isolated using previously described methodology and they were characterized as mesenchymal stem cells (positive for CD90, CD166, CD29, and negative for CD45 and CD31). Cells were seeded (10^5 cells) onto 1cmX0.5cm AlloDerm membranes 24 hours before the surgery. Four membranes: one associated with DPSC, one associated with EPMDSC, one associated with ASC, and one control (AlloDerm alone) were implanted subcutaneously on the dorsal side of athymic mice. The mice were euthanized nine days after the transplantation and H&E, imunohistochemistry for hematopoietic precursors (CD34), and imunofluorescence for human nuclei were performed to assess revascularization and the presence of the human cells in the new tissue. Results: Revascularization using AlloDerm was confirmed. Additionally, the association of mesen-

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chymal cells to this scaffold can increase the revascularization. Two sources of stem cell, ASC and EPMDSC, are the most potent cells to be associated with AlloDerm and increase the vascularization. Conclusions: Our findings show that mesenchymal stem cells seeded on alloderm increase the vascularization and that the best sources of stem cells are adipose tissue and levator palate muscle derived stem cell. Since levator palate muscle derived stem cells can be obtained from palatal surgery this can be the best mesenchymal stem cell to be associated with alloderm and increase the vascularization potential to be use in cleft lip and palate patients because levator palate muscle is a non invasive source of stem cell.

Poster Board Number: T-3184

ESTABLISHMENT OF IMMORTALIZED HUMAN ERYTHROID CELL LINES ABLE TO PRODUCE ENUCLEATED RED BLOOD CELLS

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Red blood cell (RBC) transfusion is one of the most standard transplantations. However, the shortage of RBCs for transfusion has been a serious problem. In vitro production of RBCs offers a potential means to solve this point and provides the additional advantage of freedom from infection or contamination by microorganisms. Hence, such in vitro production may become a standard procedure for generation of RBCs for clinical use in the future. We previously reported on the establishment of immortalized mouse erythroid progenitor cell lines able to produce enucleated RBCs. Here, we introduce a promising protocol for establishing immortalized human erythroid progenitor cell lines from iPS cells or umbilical cord blood stem/progenitor cells. These immortalized cell lines express erythroid-specific markers and many of the markers are upregulated after induction of differentiation in vitro. Most importantly, all these immortalized cell lines can produce enucleated RBCs after induction of differentiation in vitro. To our knowledge, this is the first report to demonstrate the feasibility of using immortalized human erythroid cell lines as an *ex vivo* source for production of enucleated RBCs.

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DEVELOPMENT OF HIGH EFFICIENT GENE DELIVERY SYSTEM USING EPISOMAL VECTOR AND MAGNETIC NANOPARTICLES FOR PRODUCTION OF IPSCS

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The development of eukaryotic expression vectors has provided a direct and convenient way of introducing novel genetic information into cultured cells. Heterologous expression of recombinant proteins has subsequently found widespread use for overproduction of therapeutically important proteins and for studies of gene regulation. Episomal vector is the nonviral autonomous replicon that was constructed for mammalian cells, expected to be applied to cellular transforming so-called direct conversion, and/or direct reprogramming. However, the transfection efficient of episomal vector is usually low using transfection reagent such as cationic polymer, polyethylenimine (PEI). To solve this problem, we attempted the development of high efficient gene delivery sytem using episomal vector and magnetic nanoparticles (MNPs). PEI coated MNPs were able to introduce plasmid vectors and express the mRNA of the target gene with high efficiency and long term (for 7 days) in mammalian cells (MEF, TIG-1) compare with conventional methods using transfection reagent and transient force expression vector. Furthermore, we introduced iPSC inducible factors (OCT4, SOX2, cMYC, KLF4, LIN28) on episomal vector. In this result, fibroblasts were differentiated into iPSC-like colony forming cell line. To optimize transfection condition, we show some factors for higher differention efficiency. This result suggests the magnetic force applied to the magnetic nanoparticles can enhance the transfection efficiency.

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FUNCTIONAL BIOARTIFICIAL CARDIAC TISSUE GENERATED FROM MURINE AND HUMAN PLURIPOTENT STEM CELL-DERIVED CARDIOMYOCYTES - IMPROVED TISSUE FORMATION BY ASCORBIC ACID AND APPLICATION OF GROWING STRETCH

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Introduction: Acute infarction of the myocardium results in the formation of a non-contractile fibrous scar, eventually leading to end-stage heart failure. The aim of our study was to develop Bioartificial Cardiac Tissue (BCT) from pluripotent stem cells for future reconstructive therapies. For a functional contractile tissue, highly purified stem cell-derived cardiomyocytes are needed. We report the efficient generation and antibiotic-based selection of cardiomyocytes from transgenic lines of murine induced pluripotent stem cells (miPSC), human embryonic stem cells (hESC) and human induced pluripotent cells (hiPSC). All cell types were successfully implemented for cardiac tissue engineering of contractile BCTs. We investigated the role of fibroblasts, ascorbic acid and mechanical stimuli on tissue formation, maturation and functionality, which was demonstrated by direct force measurement, gene expression analysis and electrophysiology. Methods and results: In a proof-of-concept-study murine iPSC-derived pure (>99% troponin T+) 3D cardiomyocyte aggregates (Cardiac bodies) were generated from transgenic cells stably expressing a Zeocin resistance gene under control of the cardiac MYH6 promoter. Addressing the limitation of cardiomyocyte loss after single cell dissociation, intact cardiac bodies were applied for BCT generation. Notably, direct fusion of non-dissociated cardiac bodies resulted in a structurally and functionally homogenous syncytium. Continuous in situ characterization of BCTs for 21 days identified three critical factors for fusion of cardiac bodies and formation of a functional BCT: i) addition of fibroblasts and ii) ascorbic acid supplementation supported ECM remodeling and CB fusion, while iii) increasing static stretch supported sarcomere alignment and cardiomyocyte coupling. All factors together considerably enhanced contractile forces of murine BCTs. We have generated human BCTs based on antibiotic-based selected human ESC- and human iPSC-derived cardiomyocytes together with mitotically inactivated human foreskin fibroblasts in a 3-dimensional collagen matrix. Again, tissue formation and force development was strongly promoted by the fibroblast-mediated remodeling of the matrix. The resulting tissue contained aligned cardiomyocytes with cross-striated morphology, and presence of Ki67+ cardio-

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myocytes demonstrated proliferative capacity up to 21 days after tissue formation. Human BCTs showed Frank-Starling curves of an increase in their active forces in response to stretch. The maximal active forces developed were up to 8.5 mN/mm2 for hESC-BCTs and 3.2 mN/mm2 for hiPSC-BCTs on d21. Field potential recordings showed propagation of the electrical stimulus in a functional syncytium and a chronotropic response to the β-adrenergic agent isoproterenol. Conclusion: Our data demonstrate the successful generation of functional murine and human engineered cardiac tissue starting from purified populations of stem cell-derived cardiomyocytes. These constructs show cell alignment and maturation and are able to contract synchronously, generating 70-times higher forces than previously reported for engineered human cardiac tissue. This makes them suitable for cardiac disease modeling, and broadens the path towards the development of cardiac patches to be used for future regenerative and replacement therapies for the heart. This work was funded by the Cluster of Excellence REBIRTH (DFG EXC62/1).

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SMART THERMO-RESPONSIVE POLYMERS AS A TOOL TO ASSESS UNDIFFERENTIATED AND MATURE CELL RESPONSE TO SUBSTRATE STIFFNESS AND NANOPATTERNING.

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The sensitivity of mature and undifferentiated cells to substrate mechano-physical properties has been investigated by a number of independent research groups so far. The results demonstrated a clear dependency of cell behaviour and phenotype on substrate chemical composition, but also on matrix mechanical features and nanostructure. The ability of living cells to activate specific signaling pathways in response to mechanical stimuli has been recently shown to be highly cell-specific, this evidence being likely to reflect differences in their physiology *in vivo*. Indeed, human mesenchymal stem cells (hMSC) can be committed to differentiate to a specific lineage when the stiffnes of a given tissue is matched by the substrate, while tissue-resident progenitors were shown to be more sensitive to pore geometry and surface patterning in the micro-scale. Contractile cells are likely to be more responsive to substrate characteristics: in embryonic cardiomyocytes, substrate stiffness was shown to affect contractility, with tissue-like Young modulus appearing more suitable for beating activity; consistently, neonatal cardiac cell maturation was demonstrated to be impaired on stiff substrates, while the formation of functional contractile units in skeletal myotubes was found to be optimal on substrates having a tissue-like stiffness. Given the ability of cells to sense the substrate they grow on and their acknowledged capacity to convert such "feelings" in a biological behavior, the possibility that scaffolds developed for a specific *in vivo* application could interfere with host cell behavior after implantation cannot be neglected. Synthetic materials can be tailored to mimic the tissue three-dimensional organization in the micro- and nanoscale, while their mechanical properties are usually far from those of the host tissue. In the present investigation, inert poly-e-caprolactone (PCL) planar layers were manufactured to obtain thermo-responsive films displaying tuneable surface features (with Young modulus ranging from kPa to MPa values and controlled nanopattern) without changing matrix chemistry. The substrates were challenged with human mesenchymal stem cells, murine skeletal myoblasts, human normal dermal fibroblasts and neonatal murine cardiomyocytes to study the possible effects of substrate stiffness and nanostructure on such cell adhesion, survival and phenotype without changing biological cues. Dynamic esperiments demonstrated a cell-specific response to the changes in surface stiffness and nano-pattern, with hMSC displaying the highest responsivity to matrix features.

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PHYSICAL FACTORS IN DIRECTING MURINE EMBRYONIC STEM CELL DIFFERENTIATION INTO OSTEOGENIC AND CHONDROGENIC LINEAGES

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In recent years, significant advances have been made to elucidate the signal transduction pathways involved in murine embryonic stem cell (mESCs) differentiation. It has also become clear that ESCs behave differently if placed in 3-dimensional scaffolds as opposed to static culture conditions. Aside from biochemical factors, it is now apparent that extra-cellular matrices (ECM) have the potential to direct stem cell differentiation, and this effect is not only based on protein composition, but in response to factors such as ECM stiffness, intrinsic forces, and extrinsic mechanical stimuli exerted on the ESC/ECM construct. The integrin family of proteins are ideally situated to play a major role in these ESC/ECM interactions, since they are transmembrane proteins that directly bind to the ECM and initiate signalling pathways that can effect gene regulation. To date, though the regulation of mESCs to mechanical stimuli has been the focus of several studies, the function of mechanosensitive proteins remains largely undetermined. Therefore, the effect of compressive loading on mESC differentiation was investigated using chondrogenesis and osteogenesis as model systems. Furthermore, the role of Arg-Gly-Asp (RDG) dependent integrin family members in this process was investigated. The ESC/ECM constructs were prepared by resuspending 106 mESC within 3D collagen I gels. To identify the role of RGD-dependent integrins, RGD peptide (cyclo Arg-Gly-Asp-d-Phe-Cys) was added to mESCs cultured under static conditions. To examine the effect of cyclic compressive loading, a FX-4000™ Compression Plus System was modified and the ESC/ECM constructs were subjected to a loading regime of 1Hz and 5% strain for 40 hours with or without the RGD blocking peptide. The morphology, proliferation rate, viability and gene expression were examined. RGD had a significant effect on cellular morphology in static culture. Cells showed a tendency to detach and to form cell aggregates, most apparent at a concentration of 0.5 mM RGD. Treatment with RGD did not significantly influence cell viability and proliferation rate in comparison to non-treated cells. At an early stage of differentiation, cyclic compression down-regulated the pluripotent genes Sox2, Rex1, and Nanog, but not Oct4. In addition, when the integrin-blocking RGD peptide was introduced at the same time points, a similar gene expression response was observed that was independent of the loading regime. Interestingly it was found that compressive loading induced chrondogenesis but not osteogenesis by day 15 of differentiation. Furthermore, the addition of RGD suppressed OCN and Col2 gene expression but up-regulated Sox9. When integrin gene expression was examined, it was observed that mechanical stimuli upregulated the collagen-dependent (α1, β1) and RGD-dependent (αv, β3) integrin subunits on day 1 of differentiation. By day 15, however, the αv subunit was up-regulated and the α1, β1, β3 subunits were down-regulated, with the addition of RGD inhibiting this effect at all stages of differentiation. Overall, these ESC/ECM constructs and RGD supplementation provide a

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model system to study the response of mESCs to physical factors and have contributed to a better understanding of integrin signalling in mESC mechanotransduction pathways.

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IS FIBRIN GLUE ESSENTIAL FOR ATTACHMENT OF HYPERDRY AMNIOTIC MEMBRANE PATHES OVER THE BONY SURFACE OF MASTOID CAVITIES IN CANAL WALL DOWN TYMPANOPLASTY?

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Fibrin glue is usually used as a reliable bioadhesive of the grafting materials in ear surgery. Because of adhering well to wound, the human amniotic membrane (AM) has been widely used clinically for membranous allograft in the field of dermatology without fibrin glue. In this study, the feasibility of the AM fixation without fibrin glue on the bony surface of mastoid cavity was evaluated in canal wall down (CWD) tympanoplasty. In 11 ears of 11 patients, the AM was attached over the bony surface of the mastoid cavity using fibrin glue (FG(+) group). In 7 ears of 7 patients, the AM was simply attached over the bony surface of the mastoid cavity without fibrin glue (FG(-) group). The times for graft epithelization were compared in both groups. In both groups, complete epithelization of the mastoid cavity took place in all patients. The mean time of epithelization in the FG(+) group was 32.4 days, while in the FG(-) group it was 31.9 days. Complete epithelization of the AM graft was not significantly different between two groups. It is suggested that fibrin glue is an inessential adhesive for attachment of the AM in the CWD tympanoplasty.

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IMPLANTATION OF BONE MARROW-DERIVED CELLS INTO IRRADIATED RAT URINARY BLADDERS RECONSTRUCT FUNCTIONAL SMOOTH MUSCLE LAYERS AND NERVE FIBERS

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Aims: We investigated to determine if bone marrow-derived cells implanted into radiation-injured rat urinary bladders could reconstruct tissue structures and recover bladder functions. Methods: Recipient female Sprague-Daweley (SD) rats were anesthetized and covered with an iron shield except for a 1-cm diameter circle bordering at the pubic bone to allow radiation of the pelvic region containing the urinary bladder. The region was radiated with 2 Gy once a week for 5 weeks, and then kept for 2 weeks. Bone marrow cells were harvested from femurs of donor male GFP-transfected SD rats and cultured for 7 days. Two weeks after the last radiation exposure, the adherent, proliferating bone marrow-derived cells were implanted into the irradiated urinary bladders (n=10). For controls, cell-free solutions were similarly injected (n=10). Four weeks after cell implantations or control injections, cystometric, histological, and immunohistochemical investigations were performed. Results: Just prior to implantation, irradiated urinary bladders showed that smooth muscle layers and nerve fibers were disorganized. The proportions of smooth muscle layer (8±1%) and nerve fiber (3±1%) areas were significantly decreased compared to un-irradiated urinary bladders (20±1%, 12±3%, respectively). In the cystometric investigations, the voiding interval of irradiated rats was irregularly prolonged, 7.92±1.09 min, and the residual volume, 0.13±0.03 ml, was significantly higher compared to the sham-irradiated rats (5.50±0.43 min and 0.05±0.01 ml). After 4 weeks, smooth muscle layers and nerve fibers in the cell-free control urinary bladders remained similar to the pre-implanted irradiated urinary bladders; however, the cell-implanted urinary bladders contained reconstructed smooth muscle layers and nerve fibers in amounts similar to the sham-irradiated urinary bladders. The proportions of the smooth muscle layer (19±1%) and nerve fiber (9±1%) areas in the cell-implanted urinary bladders were significantly higher than that in the cell-free injected controls (11±0.4%, 1±0.3%, respectively). Some GFP-positive implanted cells differentiated into smooth muscle and nerve cells and formed the reconstructed tissues. The voiding interval of cell-implanted rats, 5.46±0.33 min, was regular and similar to the un-irradiated rats, and less than the cell-free injected controls, 7.39±0.54. The residual volume, 0.04±0.01 ml, was decreased compared to the cell-free injected controls, 0.15±0.05 ml, and was similar to the un-irradiated rats. Conclusions: The bone marrow-derived cells implanted into radiation-injured urinary bladders reconstructed tissue structures. Some of the implanted cells differentiated into smooth muscle or nerve cells and formed the reconstructed tissues. The cell-implantation re-acquired regular voiding intervals and low residual volume.

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HEPATIC CELLS: AN ALTERNATIVE SOURCE TO REPOPULATE THE BIOARTIFICIAL LIVER

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Introduction: Severe hepatic failure is the result of long-term liver injury. Liver transplantation is the only efficient treatment, but is currently limited by organ shortage. The demand for new livers continues overcoming the availability. In this context, the creation of a bioartificial liver might solve this clinical problem. Aims: This work aims to produce a 3D whole-liver scaffold with intact components of extracellular matrix (ECM) and vascular system and also evaluating the repopulation of this ECM using hepatic cells. Methods: Wistar rats were used for liver excision surgery. Twenty minutes before this procedure, heparin was administered. Livers were perfused through the portal vein using an infusion pump at 4 mL/min with cold saline for 1 hour. Then, livers were washed with PBS for 30 minutes. Subsequently, livers were perfused with Triton X-100 1% for 30 min, and then with SDS 1% for 36h. After complete decellularization, livers were washed with distilled H_2O for 15 min, Triton X-100 for 30 min to remove residual SDS and then were preserved at 4ºC for 7 days. To analyze the ECM integrity post decelularization, DAPI, H&E and sirius red stainings and electronic scanning microscopy and immunofluorescence to detect collagen type I, III, albumin, laminin, osteopontin and fibronectin, were performed. Toluidine blue was used to examine the vasculature. For recellularization, cells were obtained from normal livers. These cells were cultured over slices of the decellularized liver in a 24-well plates and immunofluorescence assays to detect albumin, HNF and CK-18 were performed. Results: Toluidine blue showed that the vascular system was totally preserved. Macroscopy, microscopy and histological staining showed that the decellularization process preserves the structure and components of the ECM. After 30 days,

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cells were detected in the decellularized tissue. Conclusions: Our decellularization method was efficient in removing resident cells and preserving the liver's ECM and vascular system. Moreover, liver cells were able to adhere on liver 3D scaffold generating new perspectives for building a bioartificial liver.

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COMPLETE DENTAL PULP REGENERATION BY TRANSPLANTATION OF PULP STEM CELLS WITH G-CSF

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Dental pulp is critical for homeostasis of teeth, regeneration of pulp following root canal treatment in pulpitis and resultant improvement of dental function. The long term goal of endodontic treatment following deep caries and/or pulp inflammation is the conservation and restoration of teeth including dental pulp. A promising approach is stem cell therapy to regenerate the dentin-pulp complex for the conservation and total restoration of structure and function of teeth. We succeeded in complete pulp regeneration after transplantation of dental pulp CD105+ or CD31- SP stem cells with high angiogenic and neurogenic potential into pulpectomized teeth with stromal cell-derived factor 1 (SDF1) in dogs. CD31- SP cells are isolated after labeling with DNA binding dye Hoechst 33342 and flowcytometry. CD105+ cells are isolated by magnetic antibody beads method and have to be cost effective. The unfractionated total pulp cells by a single colony method are not optimal for pulp regeneration since the regenerated tissue is less in volume compared to fractionated stem/progenitor cells. Thus, we have developed novel isolation methods ensuring safety and efficiency from small amounts of pulp tissue to permit clinical trials. In addition to stem/progenitor cells, migration/homing factors should be produced in GMP facilities for clinical investigation. In the present study we have examined granulocyte colony-stimulating factor (G-CSF), a widely used hematopoietic stem cell mobilization cytokine, as a cell migration factor instead of SDF-1. The isolated dental pulp stem cells by our novel method highly expressed stem cell markers, angiogenic/neurogenic factors compared with unfractionated total pulp cells. In addition they exhibited potent chemotactic and mitogenic activities and multilineage differentiation potential including vasculogenic and neurogenic lineages. Autogenous transplantation of the pulp stem cells with G-CSF were performed after pulpectomy in dogs. The root canal was filled with regenerated pulp tissue including vasculature and nerves by day 14. The odontoblast-like cells attached to the dentinal wall in the root canal, and produced dentin-like tissue extending their processes into dentin tubules by 60 days. The transplanted pulp stem cells had high migratory and proliferative effects due to G-CSF. Angiogenic/neurotrophic factors were expressed by the G-CSF-treated cells and they localized in the vicinity of newly formed capillaries, suggesting possible trophic effects. Transplantation of pulp stem cells with G-CSF yielded significantly more regenerated tissue compared with transplantation of stem cells alone or G-CSF alone. When unfractionated total pulp cells were transplanted, less tissue was observed compared with pulp stem cells, followed by evidence of matrix formation and mineralization on day 60. It is noteworthy that the regenerated tissue demonstrated expression of authentic dental pulp cell markers. In conclusion, dental pulp stem cell therapy with G-CSF is of utility for pulp regeneration following pulpectomy, demonstrating safety and efficacy to permit clinical applications in the near future.

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REGENERATION OF HOLLOW ORGANS A PLATFORM APPROACH

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Recent successes in regenerative medicine and tissue engineering of hollow organs have leveraged regenerative constructs composed of: (1) a biodegradable, custom fabricated scaffold based on synthetic, biopolymers approved by regulatory agencies (2) a population of smooth muscle-like cells. As acellular constructs do not mediate regenerative outcomes, cell sourcing is fundamental for hollow organ engineering. Mesenchymal stem cells (MSC) are under active investigation for applications in regenerative medicine, but may not be ideal for industrial-level organ engineering. We demonstrate that smooth muscle-like cells (SMC) for hollow organ regeneration are isolatable from cell sources alternate to the primary organ. Transcriptional, proteomic and functional methodologies identify SMC as a cellular sub-population with markedly distinct phenotypic and functional properties relative to MSC. We have demonstrated that implantation of regenerative constructs within human or animal subjects catalyzes the de novo regeneration of a neo-tissue/organ. We provide further evidence from multiple organ systems that this methodology represents a broadly applicable organ regeneration platform for tissue engineering multiple, disparate laminarly organized hollow organs. Specifically: 1) Esophagus. Using rodent models of esophageal injury, we demonstrate that patch regenerative constructs based on this platform technology mediate complete regeneration of esophageal wall by 10 weeks post-implant in rodents. These results are foundational for on-going attempts to regenerate tubular esophageal constructs in pre-clinical large animal models and eventually for clinical trials in human patients requiring esophageal replacement secondary to esophageal cancer. 2) Small intestine. Leveraging rodent models of small intestine injury, we show that patch and tubular regenerative constructs mediate regeneration of small intestine tissue with laminarly organized neo-mucosa and smooth muscle layers as early as 8 weeks post-implant in rodents. Follow-up studies with pre-clinical large animal models will lay the framework for clinical trials in human subjects presenting with short bowel syndrome.
3) Urinary conduit. In a pre-clinical GLP study, we demonstrate that implantation of tubular regenerative constructs with SMC sourced from autologous adipose or peripheral blood into a porcine cystectomy model triggers de novo regeneration of a hollow neo-organ composed of urinary-like neo-tissue histologically identical to native bladder. The neo-urinary conduit (NUC) represents the first of a class of entirely novel neo-organs that have hitherto not existed in nature. Remarkably, this engineered neo-organ functions as expected in vivo to efflux urine. Tengion is currently conducting Phase I clinical trials of NUC constructs in human patients requiring urinary bypass secondary to radical cystectomy as a consequence of bladder cancer. Unlike other tissue engineering approaches, this organ regeneration platform does not require use of cadaveric organs or stem cell populations potentially recalcitrant to industrial scale process development. Taken together, we believe that the ability to create hollow organs de novo from biodegradable, synthetic scaffolds seeded by autologous SMC sourced alternate to the primary organ will greatly facilitate translation of hollow organ tissue engineering technologies into clinical practice.

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DIFFERENTIAL ANGIOGENESIS, NEUROGENESIS AND PULP REGENERATION FROM PORCINE CD31$^-$ SIDE POPULATION CELLS ISOLATED FROM DENTAL PULP, BONE MARROW AND ADIPOSE TISSUE

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Background: Pulp CD31- side population (SP) cells have high angiogenic, neurogenic and pulp regeneration potential. However, decrease in the abundance and volume of pulp tissue with age makes the pulp stem cell therapy and its clinical utility challenging. In this study, we investigated the comparative regenerative potential of porcine pulp, bone marrow and adipose tissue-derived CD31- SP cells to evaluate an alternative cell source for pulp regeneration. Methodology/Principal Findings: The primary porcine pulp cells, adipose cells and bone marrow cells were separated from the same individuals, and were labeled with Hoechst 33342 and further anti-porcine CD31. The frequency of CD31- SP cells in pulp, bone marrow and adipose tissue was 0.9%, 0.3% and 0.1%, respectively. Flow cytometric analyses of cell surface antigen markers demonstrated the "stemness" of bone marrow and adipose CD31- SP cells as pulp CD31- SP cells. Bone marrow and adipose CD31- SP cells, however, had less expression of angiogenic/neurotrophic factors and less migration activities compared with pulp CD31- SP cells. The angiogenic and neurogenic potential was similar in the three cell populations. In mouse hindlimb ischemic models, transplantation of bone marrow and adipose CD31- SP cells resulted in significantly less increase in the blood flow and density of capillary formation compared with pulp CD31- SP cells. In rat brain ischemic models, recovery of motor function and decrease in infarct size were less in bone marrow and adipose CD31- SP cells compared with pulp CD31- SP cells. In tooth root models of subcutaneous transplantations, bone marrow and adipose CD31- SP cells induced vasculogenesis, neurogenesis and pulp regeneration with biomarker expression of syndecan and thyrotropin-releasing hormone degrading enzyme (TRH-DE), although yielded less regenerated tissue compared with pulp CD31- SP cell transplantation on day 28. The GOT-stained porcine transplanted cells were not co-localized with RECA1 positive capillaries in all the three transplantations. The transplanted cells were not stained with Ki67 on day 28, indicating little proliferation. Some of the cells in the regenerated tissues were not stained with GOT. Highly expression of angiogenic/neurotrophic factors, such as vascular endothelial growth factor-A (VEGF-A), matrix metalloproteinase (MMP)-3, granulocyte-monocyte colony-stimulating factor (GM-CSF), brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) in the transplanted cells were found by in situ hybridization on day 28. Conditioned media from bone marrow and adipose CD31- SP cells showed similar angiogenic, neurogenic and chemotactic activities on NIH3T3 cells *in vitro* as that from pulp CD31- SP cells. Conclusion/Significance:These results suggested potent trophic effects of bone marrow and adipose CD31- SP cells as pulp CD31- SP cells to enhance homing of stem/progenitor cells in the tooth roots, contributing to pulp regeneration. Thus, bone marrow and adipose CD31- SP cells may be alternative cell sources of clinical utility for pulp regeneration, although its regenerative potential is inferior to pulp CD31- SP cells.

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REGENERATING KIDNEY STRUCTURE AND FUNCTIONALITY BIORESPONSE OF RODENT MODELS OF RENAL DISEASE TO IMPLANTATION OF NEOKIDNEY AUGMENT PROTOTYPES COMPOSED OF SELECTED RENAL CELLS AND BIOMATERIALS

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Chronic kidney disease (CKD) is continued loss of renal function over time. Current renal therapies include dialysis and kidney transplant. An unmet need exists for new treatments to restore renal function thus delaying dialysis/transplant. Efforts to regenerate diseased kidneys have focused on application of mesenchymal stem cells (MSC). However, we have identified populations of tubular epithelial cell-enriched primary renal cells (Selected Renal Cells, SRC) that positively impact aspects of disease phenotype in rodent CKD models, potentially by interfering with onset of tubulointerstitial fibrosis and mobilizing host renal stem cell populations. Addition of biomaterials may facilitate cellular engraftment while creating space for vascularization and cellular infiltration during regeneration. Here, we report on development of Neo-Kidney Augment (NKA) product prototypes, comprised of SRC/biomaterials combination constructs which facilitate regeneration of kidney tissue. Methods: Identification of biomaterials appropriate for renal tissue engineering was accomplished by iterative screening and evaluation of bio-response to implantation of SRC/biomaterial constructs within rodent models as follows: (1) Healthy adult rodents (n=200) were used to identify biomaterials associated with minimal inflammatory and fibrotic outcomes, cellular infiltration and neo-kidney tissue induction (2) Removal of single kidney from rodents (hemi-nephrectomy) facilitates detection of systemically acting toxicological effects. Hemi-nephrectomized rodents (n=20) were injected with SRC/biomaterial NKA prototypes within renal parenchyma of the remnant kidney. Physiological indices derived from whole blood, serum and urine chemistries were evaluated at 2 and 4 week time points post-implantation. Animals were sacrificed at 4 weeks post-injection and remnant kidney examined histologically for evidence of inflammatory or fibrotic bio-response (3) Removal of single kidney and 5/6 of remnant kidney induces disease physiologies within rodents mimicking aspects of chronic kidney disease (CKD) in humans, permitting evaluation of bio-response to implantation of SRC/biomaterials NKA prototypes within a clinically relevant small animal model. 5/6 nephrectomized rodents (n=20) were implanted and evaluated as described above for hemi-nephrectomized rodents. Results: Of the biomaterials candidates examined, implantation of gelatin-based hydrogels within healthy rodent renal parenchyma was associated with minimal fibrotic and inflammatory outcomes and concomitant neovascularization, cell and tissue infiltration and biomaterial degradation within 4 weeks post-implantation; by 8 weeks, neo-kidney tissue was observed, suggesting regenerative response induction in vivo. Implantation of SRC/biomaterials NKA prototypes within remnant renal parenchyma of hemi- or 5/6- nephrectomized rodent kidneys presented minimal evidence of inflammatory, necrotic or fibrotic bio-response and did not significantly affect key renal physiological indices.

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Therefore, NKA prototypes based on SRC/gelatin-based hydrogels are well tolerated by remnant kidney in rodent hemi- or 5/6- nephrectomy models. Conclusions: Together with previous studies, these data establish SRC/gelatin-based hydrogel NKA prototypes for tissue engineering of CKD kidneys. Evaluation in canine CKD models is in progress prior to start of GLP animal studies.

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HEPATOGENIC DIFFERENTIATION OF MENSTRUAL BLOOD DERIVED STEM CELLS ON A THREE DIMENSIONAL NANOFIBROUS SCAFFOLD

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Introduction:In few recent years, such problems with well-known stem cell sources as low availability, painful access or limited proliferative ability have impelled scientists to take advantages of menstrual blood stem cells (MenSCs) in tissue engineering and regenerative medicine. However, only little information is available about hepatogenic differentiation potential of MenSCs. In the present study, we examined the differentiating potential of MenSCs into hepatocytes on a three dimentional nanofibrous scaffold fabricated from Poly (ε-caprolactone) (PCL). Methods: MenSCs were isolated of menstrual blood samples by discontinuous density gradient centrifugation and plastic adherence. After characterization of seeded MenSCs on matrigel coated PCL nanofibers, differentiation ability of cultured cells into hepatocyte using a novel three-stage protocol was evaluated by biochemical and molecular experiments. Results: Flow cytometric analysis illustrated that MenSCs were strongly positive for mesenchymal and negative for hematopoietic stem cell markers. MenSCs also exhibited marked expression of OCT-4. Based on the result of scanning electron microscopy, MTT assay and Hematoxilin & Eosin staining, cells adhered, distributed and expanded on the scaffold. The cultured MenSCs on scaffold in presence of hepatic conditioned media significantly expressed mature hepatocyte mRNA such as albumin, tyrosine amino transferase and cytochrome P7A1 on day 30 of differentiation judged by RT-PCR. Moreover, unlike undifferentiated MenSCs, great synthesis and production of albumin protein by differentiated MenSCs was shown by immunofluorescent staining and enzyme-linked immunosorbent assay using monoclonal antibody against albumin protein.Conclusion: The fabricated scaffold supports MenSCs differentiation into hepatocyte-like cells. So, a new stem cell-scaffold construct has been suggested for treatment of liver diseases using hepatic tissue engineering

Totipotent/Early Embryo Cells

Poster Board Number: T-3197

EMERGENCE OF PLURIPOTENT STEM CELLS CONTROLLED BY KLF5

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In our previous study, we generated Klf5 knock-out mice and showed that Klf5 is indispensible for blastocyst development and the derivation of ESCs from the inner cell mass (ICM)(Ema et al., 2008). Recent studies indicate that Kruppel-like factors (Klfs) are essential for both maintenance of ES cell self-renewal and reprogramming of somatic cells into a pluripotent state. However, the molecular mechanism underlying these functions remains unknown. To understand the mechanism, we first addressed the precise phenotype of Klf5 KO embryos. By investigating the BrdU incorporation activity and the pluripotency-related and lineage-marker expressions in Klf5 KO embryos at each early development stage, we show that cell cycle progression is impaired before the reduced cell number and lineage marker expression appeared. Importantly, level of expressions for Nanog, Oct3/4, and Cdx2 are comparable in WT embryos and Klf5 KO embryos with same cell number, indicating that reduced Nanog, Oct3/4, and Cdx2 expression seen in Klf5 KO embryos are due to the growths retardation of Klf5 KO embryos. These results suggest that there are common mechanisms in the regulation of cell-cycle between mESCs and early mouse embryonic cells. To reveal the cell-cycle regulation mechanism by Klf, we amplified the cDNA from embryos and performed the microarray analysis. Then we found that intracellular signaling pathway A was activated in Klf5 KO embryos. Pharmacological inhibition for signaling pathway A by specific inhibitor resulted in significant rescue for Klf5 KO phenotypes, such as the growths retardation and apoptosis. We conclude that Klf5 suppress intracellular signaling pathway A and act as a safeguard for the normal cell-cycle progression and differentiation.

Poster Board Number: T-3198

TRANSCRIPTION FACTOR DYNAMICS ON THE SOX-OCT CIS REGULATORY ELEMENTS EXPLAIN THE EARLY MOUSE EMBRYO DEVELOPMENT

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Oct4 and Sox2 regulate the expression of target genes such as *Nanog, Fgf4*, and *Utf1*, by binding to their respective regulatory motifs. Their functional cooperation is reflected in their ability to heterodimerize on their adjacent cis-regulatory elements, the composite sox-oct *cis*-regulatory element (SO-CRE). Given the many developmental genes regulated by Oct4 and Sox2, a quantitative analysis of their apparent cooperativity would yield valuable insight into mechanisms of early embryonic development. In this study, we measured the apparent cooperativity of Oct4 and Sox2 on SO-CRE using fluorescence correlation spectroscopy (FCS). We found that interaction is driven mainly by the Sox2 concentration instead of Oct4 in the case of *Fgf4*'s SO-CRE. Taking into account that *Sox2* expression levels fluctuate more than *Oct4*, our finding could explain how Sox2 controls epiblast (EPI) and primitive endoderm (PE) cell fate decision within the developing blastocyst.

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iPS Cells

Poster Board Number: T-3201

DEVELOPMENT OF EFFICIENT INDUCTION METHODS FROM HUMAN IPSCS/ESCS INTO INTERMEDIATE MESODERM BY USING LOW MOLECULAR WEIGHT COMPOUNDS

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The increased prevalence of chronic kidney disease has caused a rise in the number of dialysis patients, and is associated with elevated morbidity and mortality. Development of kidney regeneration therapy is required because most patients with chronic kidney disease never recover renal function. Kidney is derived from one of the early embryonic germ layers, intermediate mesoderm (IM), and directing pluripotent stem cells, such as induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs), into IM lineage is a crucial step for kidney regeneration. In this study, to identify small molecules that can efficiently induce the differentiation of human iPSCs/ESCs into IM lineage cells, we screened a chemical library of about 1,800 low-molecular weight compounds using human iPSC lines that contain an allele of an IM-specific marker gene OSR1 into which a green fluorescence protein (GFP) gene was knocked-in by homologous recombination. We identified two chemical compounds with the inducing ability and established a differentiation protocol using the combinational treatment of these compounds that directs the differentiation of human iPSCs/ESCs into IM lineage cells. The combinational treatment with these compounds induces nearly 90% of OSR1$^+$ cells from human iPSCs for only 6 days, in the same way as that achieved by our combinational treatment of growth factors for 11 days. Moreover, the generated OSR1$^+$ cells produced the substantial expression of other IM markers, and had the ability to differentiate into the cells expressing the specific markers for renal cells constituting the adult kidney *in vitro* and *in vivo*. Furthermore, we elucidated a part of the mechanisms involved in the differentiation of human iPSCs/ESCs into IM lineage cells. In conclusion, we have identified two low-molecular weight compounds that can induce a robust differentiation of human iPSCs/ESCs into IM cells with the similar developmental potential to their *in vivo* counterpart. Our differentiation methods using chemicals alone can produce faster, more consistent and lower-cost generation of IM cells than growth factor-based protocols, which may contribute to understanding the mechanisms of kidney development and supplying the cell sources for the regenerative medicine strategies for chronic kidney disease.

Poster Board Number: T-3202

EFFICIENT GENERATION OF HUMAN INDUCED PLURIPOTENT STEM CELLS IN A FEEDER-FREE AND XENO-FREE CONDITION

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Due to an extraordinary speed of development in the iPSC research, we know that iPSCs share many key properties with ESCs including pluripotency, self-renewal, morphology, colony formation, and gene expression profiles. These similar traits of iPSCs to ESCs are thought to lead the cells to clinical applications in the foreseeable future. One important safety-related issue for clinical application of iPSCs is the generation and expansion of the cells in the absence of animal-derived products in the media. It was reported that hESCs incorporated significant amount of nonhuman sialic acid, Neu5Gc, in the cell surface proteins which may result in failure in transplantation due to immune rejection. Furthermore, iPSCs cultured in animal-derived products can be a source for non-human pathogen transmission to human. In that sense, conventional mouse embryonic fibroblast (MEF)-based culture methods are not suitable for clinical application. In this study, we were able to generate hiPSCs efficiently in a feeder-free & xeno-free culture condition. Our study provides valuable information which would expedite clinical application of human pluripotent stem cells to treat many incurable diseases. *This research was supported by grant SC5170 from the Stem Cell Research Center of the 21st Century Frontier Research Program, and Stem Cell Research Program (2010-0020350) funded by the Ministry of Education, Science and Technology of Republic of Korea.*

Poster Board Number: T-3203

'THREE-IN-ONE' SENDAI VIRUS VECTOR IS HIGHLY PROMISING FOR EFFICIENT GENERATION OF TRANSGENE-FREE HUMAN IPS CELLS.

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[Abstract] Induced pluripotent stem cell (iPSC) technology is a promising technology for drug discovery and regenerative medicine. It is important to select an efficient and safer gene transfer vector for the generation of high-quality iPS cells. Previously, we reported efficient generation of transgene-free, vector-free iPS cells using Sendai virus (SeV) vector, a non-integrating cytoplasmic RNA vector, carrying the OCT3/4, SOX2, KLF4 and c-MYC gene, independently. To further increase the efficiency, we created SeV vectors that carried three genes encoding reprogramming factors per vector and tested the gene order to examine the efficiency of iPSC induction. Methods and Results: The order of the genes for the generation of iPSCs: KLF4-OCT3/4-SOX2 (SeV-KOS) and OCT3/4-SOX2-KLF4 (SeV-OSK) in combination with c-MYC (SeV-c-MYC) were examined, because the expression level is affected by the gene order. We found that the efficiency was up to 4% by using SeV-KOS when we used BJ human neonatal fibroblast cells as a source of parental cells. It was ten times higher than that by SeV-OSK or previous vector constructs using four genes cocktail. However, the efficiency was much improved when SeV-OSK vector was used together with additional SeV vector carrying KLF4 (SeV-OSKK). These results suggested that a certain high level of KLF4 expression was necessary for the efficient iPSC generation. Next, we created a temperature-sensitive SeV vector carrying KOS (SeV-KOS/TS12). We tested the efficiency of iPSC generation together with two kinds of SeV-c-MYC vectors (SeV-c-MYC/TS12 and SeV-c-MYC/TS15, respectively). TS15 is more temperature-sensitive than TS12. When we used a combination of SeV-KOS/TS12 and SeV-cMYC/TS12, iPSCs were generated efficiently (up to 3%) at 37°C and vectors were removed by culturing at 39°C for a week. When SeV-KOS/TS12 and SeV-c-MYC/TS15 were used, iPSCs were generated efficiently at 36°C (up to 2%) and iPSCs became vector-free by culturing at 37°C within almost 5 passages. We confirmed that iPSCs induced by using SeV-KOS and OSKK showed normal karyotype and had similar properties of ES cells such as pluripotency and ES marker expression. Conclusion: These results suggest that SeV vectors carrying three genes encoding reprogramming factors in one vector backbone is a promising vector to efficiently generate tansgene-

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and vector-free iPSCs. Acknowledgements: This work was in part supported by JST, PRESTO.

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REGULATORY CIRCUITS COMMON TO HEMATOPOIETIC AND EMBRYONIC STEM CELLS DIRECT BULK NON-INTEGRATED REPROGRAMMING OF LINEAGE-COMMITTED HUMAN MYELOID PROGENITORS

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Factor-driven reprogramming of human somatic cells using non-viral, non-integrating methods is slow and inefficient. We previously reported the derivation of non-integrated, transgene-free cord blood-derived induced pluripotent stem cell (CB-iPSC) lines that were generated at efficiencies between 1 - 4% of input cells using a novel bone marrow stromal cell (BMSC) co-culture system and an EBNA-based episomal system. In designing this BMSC-CD34+ co-culture reprogramming system, we capitalized on the principle that the innate epigenetic plasticity of hematopoietic progenitors can be positively influenced by stem cell niche signals. We now report the optimized non-integrated reprogramming of lineage-committed CB myeloid progenitors with a single plasmid episome expressing only the four Yamanaka factors (SOX2, OCT4, KLF4, MYC), and with unprecedented bulk efficiencies up to 50%. Contrary to prior studies that suggested stem-progenitors are more amenable to reprogramming, lineage-committed CD33+CD38+CD45+CD34- myeloid cells, and not primitive CD34+ hematopoietic stem-progenitors (HSPC) were the main targets of this highly permissive and nearly complete (60-80% NANOG+TRA-1-81+) episomal reprogramming. When myeloid progenitors were FACS-purified from HSPC populations, CD34+CD38hiCD33+ myeloid progenitor cells (16.7±3.7%) revealed significantly more amenable to pluripotency induction compared to CD34+CD38low HSPC populations (0.33±0.3%). The true reprogramming efficiency of myeloid progenitors was assayed by enumerating alkaline phosphatase+ TRA-1-81+ hiPSC that emerged from plasmid-nucleofected CB cells, and by enriching only for hematopoietic cells that had been successfully transfected via GFP co-nucleofection. These studies revealed the reprogramming efficiency of lineage-committed GFP+CD34-CD45+ myeloid progenitor that was 51±10.5%. Further mechanistic studies revealed that the conversion of myeloid populations into NANOG+TRA-1-81+ iPSC was directed en masse by a synergy between the Yamanaka factors (SOKM), hematopoietic growth factors, and extrinsic stromal micro-environmental signals. Highly efficient pluripotency induction correlated not to endogenous expression of core pluripotency factors (e.g. OCT4, SOX2, NANOG) in myeloid cells, but instead to *de novo* expression of transcriptional circuits that commonly regulate epigenetic plasticity and self-renewal in both hematopoietic progenitors and embryonic stem cells (ESC). Using a modular bioinformatics approach, we observed that CB myeloid progenitors expressed activated ESC-like levels of the OCT4 interactome and MYC-regulated circuits, and inactivated ESC-like Polycomb group (PcG)-regulated networks that were poised *de novo* in partially-reprogrammed states. These findings solve the problem that hiPSC emerge only from rare factor-expressing somatic cells, and open the cellular biology of large synchronized populations of reprogrammed cells to full kinetic analysis. These data also provide the first evidence that induced pluripotency may require activation of a molecular framework that commonly regulates self-renewal and differentiation in both hematopoietic progenitors and ESC, and that is already expressed at high, partially-reprogrammed levels in myeloid progenitors.

Poster Board Number: T-3205

GENETICALLY IDENTICAL HUMAN EMBRYONIC AND INDUCED PLURIPOTENT STEM CELLS REVEAL SIGNIFICANT DIFFERENCES IN DIFFERENTIATION CAPABILITIES TO MESODERMAL LINEAGES

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It has been extensively evidenced that both hESC and hiPSC have the capability of differentiating into many different cell types of the three germ layers. However, it has recently emerged upon their further scrutiny, that hESC and hiPSC exhibit significant differences in their comparative differentiation capabilities and epigenetic properties. It is hypothesized these differences may be due to the fact that each cell line reported thus far has been derived from genetically individual sources. In an attempt to identify the underlying cause of the differences observed between hESC and hiPSC, we have terminally differentiated hESC to definitive fibroblasts then reprogrammed these cells to hiPSC, creating genetically identical, or isogenic, hESC and hiPSC. Upon characterization of the generated hiPSC lines, we examined the mRNA expression and DNA methylation patterns of all cells in their undifferentiated state. We then differentiated these cells, in parallel with the parental hESC from which they were derived, to cells of the mesodermal lineage, specifically cardiac and hematopoietic cell types. Cardiomyocytes and hematopoietic cells were then compared with respect to their mRNA and protein expression patterns. Our results showed there were significant differences in differentiation capacities and expression patterns, even though in an undifferentiated state, the DNA methylation and pluripotent gene expression levels were found to be highly similar. This suggests that other factors, such as somatic cell source and/or the process of reprogramming itself, creates the observed differences between hiPSC and hESC. The creation of hiPSC derived from isogenic sources is a novel way to pinpoint differences in hiPSC vs. hESC not related to genetic background.

Poster Board Number: T-3206

IMMORTALIZED HUMAN FORESKIN FIBROBLAST FEEDER CELLS WITH ENDOGENOUS SECRETION OF BASIC FGF SHOWS UP-REGULATION OF TGF BETA AND IGFII : KEY FACTORS OF HUMAN EMBRYONIC STEM CELLS/INDUCED PLURIPOTENT STEM CELL PLURIPOPTENCY AND SELF RENEWAL

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Recently, it has been shown that several members of TGF-β family and IGF-II are necessary for the maintenance of pluripotency and self renewal in Human Embryonic Stem Cells (hESCs). Both the components have been proposed to cooperate with FGF pathway to maintain pluripotency and self renewal by establishing a regulatory stem cell niche. With the enhanced understanding of hESC culture conditions it has been found that the supplementation with b-FGF is still required to sustain pluripotency. Immortalized Human Foreskin Fibroblast (I-HFF) feeder cell line (established & kind gift

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from Prof. Anis Feki, Laboratory of Stem Cell Research, Geneva University Hospital, Switzerland) stably transduced with Bmi, hTERT, GFP and secreted form of b-FGF gene was used. The I-HFF cell lines have been successfully validated and proven to support the growth and maintence of hESC and Induced Pluripotent Stem Cells (iPSC). The present study was designed to evaluate the minimum requirement of exogenous b-FGF to be added to meet the standard regulation of key factors involved in the regulation of pluripotency compared to naive Human Foreskin Fibroblast cells used as feeders. I-HFF cells and HFF cells after mitomycin inactivation were cultured for additional 5 days using varying concentration of exogenous b-FGF at 10 ng/ml, 5 ng/ml, 2 ng/ml and 0 ng/ml for I-HFF and with 10ng/ml, standard concentration for HFF. The cultures were harvested at Day 02 (1st harvest) and Day 05 (2nd harvest), mRNA was isolated and reverse transcribed, initially the presence of FGF receptor 4 was checked with RT-PCR on both the feeders. The semi quantitative PCR was done to observe any difference in the expression of both TGF-β and IGF-II genes at all concentration of exogenous b-FGF and without b-FGF in I-HFF cells. Furthermore, using SYBR green chemistry the fold expression of both the genes was quantified using GAPDH as a reference gene for all the parameters. The fold expression was negligible when I-HFF without b-FGF was compared with I-HFF at all other b-FGF concentration used for both the genes, corresponding with the semi quantitative PCR results. Next, I-HFF was compared to the HFF without exogenous b-FGF, a 2.38 and 2.85 fold increase was observed for TGF-β and IGF-II genes respectively. Likewise, I-HFF at all b-FGF conc. and without b-FGF regime was compared with HFF at 10ng/ml b-FGF conc. in both the harvests. Interestingly, we also found a sequential expression in the fold expression of both the genes between 1st and 2nd harvest, indicative of a reproducible trend. These finding indicate the likely hood of using the conditioned media (CM) of I-HFF feeder cells in the maintenance of feeder free culture of hESC, without the variability of batch to batch feeder cell variation. This preliminary study indicates the fact that I-HFF can be a better alternative to the naive HFF and for hESC culture maintenance without additional requirement of exogenous b-FGF.

Acknowledgement: We thank Prof. Anis Feki for providing us Immortalized Human Foreskin Fibroblast Feeder cell line.

Poster Board Number: T-3207

MODELING CONGENITAL CENTRAL HYPOVENTILATION SYNDROME USING HUMAN INDUCED PLURIPOTENT STEM CELLS

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Congenital Central Hypoventilation Syndrome (CCHS) is a rare disorder of respiratory control that shows significant neural crest (NC)-pathology. CCHS is highly correlated with mutations in the *PHOX2B* gene. *PHOX2B* is a *paired*-like homeodomain transcription factor. CCHS-associated *PHOX2B* mutations show preservation of the DNA homeodomain structure, and therefore may continue to bind DNA; however, how this interaction affects gene regulation remains unknown. The ultimate goal of this project is to generate an *in vitro* model of CCHS using induced pluripotent stem (iPS) cells derived from a CCHS autopsy subject that suffered from a severe case of intestinal aganglionosis. To this end, we validated full cellular reprogramming of fibroblasts by molecular marker expression. Furthermore, we differentiated the PHOX2B mutated iPS cells into all three human germ layers.

Poster Board Number: T-3208

THE PLURIPOTENCY MARKER, ZFP42 (REX1) IS HIGHLY UPREGULATED UNDER HYPOXIC CULTURE CONDITIONS IN THE MULTILINEAGE-DIFFERENTIATING STRESS-ENDURING (MUSE) SUBPOPULATION OF PRIMARY HUMAN FIBROBLASTS.

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Hypoxic conditions are known to support both pluripotency in embryonic stem (ES) cells as well as to enhance the efficiency of reprogramming of somatic cells to induced pluripotent stem (iPS) cells. Biopsy derived fibroblasts are an easy accessible source for obtaining patient specific cells for reprogramming. However, studies have shown that the efficiency of reprogramming of such cells is very low, usually in the range of 0,01 - 0,05%. Interestingly, recent data have shown that only a small subpopulation of human dermal fibroblasts are prone to be reprogrammed into iPS cells, whereas the major part of the cells are not completely reprogrammed and thus are maintained in a partially pluripotent state. Investigations of human primary fibroblasts, mesenchymal stem cells as well as native bone marrow cells, all seem to possess a few percent of this subpopulation, which are characterized by heterogenous expression of the Stage Specific Embryonic Antigen 3 (SSEA-3), an embryonic/pluripotency marker. These cells, called MUSE (multilineage-differentiating stress-enduring) cells, can efficiently be isolated by FACS sorting as cells positive for both SSEA-3 and the mesenchymal cell marker CD105. Since MUSE cells only constitute a small subpopulation of a primary fibroblast cultures, we have initiated an investigation of culture conditions promoting the proliferation and expansion of these cells without loss of the $SSEA\text{-}3^+/CD105^+$ phenotype. Using FACS sorting we have investigated whether cultivation of primary human fibroblasts under hypoxic conditions affect the $CD105^+/SSEA3^+$ phenotype. Also, we tested the effect of the addition of bFGF on the $CD105^+/SSEA3^+$ phenotype. To obtain a better understanding of this subpopulation, we have used qPCR analysis to study how different growth conditions affect the expression of specific genes and miRNA's known to be important for reprogramming and maintenance of pluripotency. The data shows that both hypoxic conditions and addition of bFGF increase the number of $CD105^+/SSEA\text{-}3^+$ cells in culture in an additive manner. Furthermore, several genes known to be important for pluripotency and self-renewal have induced expression. Interestingly, we find that the Zinc finger protein, ZFP42 (REX1), known to be involved in self-renewal in ES cells, is highly upregulated, more than 20-fold (qPCR), in hypoxic conditions as well as by addition of bFGF.

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Poster Board Number: T-3209

INDUCED PLURIPOTENT CANCER CELLS FROM INDIVIDUAL PATIENTS AS A METHOD FOR STUDYING ONCOGENESIS OF HUMAN MALIGNANT MELANOMA.

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Melanoma is thought to arise through a series of genetic and epigenetic events. Primary it involves numerous irreversible changes within the human genome, such as chromosomal number alterations, gene amplifications and mutations. Recently, however, it has become widely recognized that epigenetic events may occur at much higher frequency compare to gene mutations and thus may have a greater impact on the process of melanoma tumorigenesis. Furthermore, several studies showed the reversion of the metastatic phenotype of aggressive cancer cells and revealed the convergence of embryonic and tumorigenic signaling pathways, which may help to identify new targets for therapeutic intervention. Generation of cancer-specific iPS cells, also called induced pluripotent cancer (iPC) cells, provides valuable experimental platform to the model of oncogenesis. In our work we have established and characterized primary melanoma cell lines from human biopsies. Established cell lines were analyzed for the endogenous expression of the pluripotency markers and then reprogrammed into ES-like state using individual ectopic expression of reprogramming factors for each primary cell line. It was demonstrated that established iPC cell lines express pluripotent stem cell markers, and, despite of transformation status, primary melanoma cell lines can be converted to the ESC epigenetic state. Established iPC cell lines could serve as a model for oncogenic transformation study and hold great potential for drug screening.

Poster Board Number: T-3210

DISTURBANCE OF A DEVELOPMENTAL SWITCH IN SCHIZOPHRENIA: A HUMAN NEURONAL CELL THROUGH SPECIFIC PHOSPHORYLATION OF DISC1

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Schizophrenia (SZ) is a severe brain disorder that results in mental dysfunctions and significant social disability in affected individuals. Deficits associated with neurodevelopment have been suggested but the molecular mechanisms remain elusive. A major obstacle to address this question directly has been in the difficulty of accessing relevant tissues/cells for study. Thus, we have systematically collected tissue/cells and established a panel of human cell lines, including olfactory immature neurons and induced pluripotent stem cells (iPSCs) from patients with schizophrenia as well as normal controls. We have generated iPS cells from skin fibroblasts from sporadic SZ patients and controls by the retroviral transduction of four factors: OCT4, SOX2, KLF4 and c-MYC. We then differentiated iPS cells to neurons via embrio body and neurosphere formation using previous method with slightly modification. Our recent study indicated that phosphorylation at a specific site [serine-710 (S710) in mouse] on DISC1 acts as a molecular switch from maintaining proliferation of mitotic progenitor cells to differentiation and migration of postmitotic neurons (Ishizuka et al, Nature, 2011). We hypothesized that deficits in the phosphorylation of human DISC1 at serine-713 (S713) that corresponds to mouse DISC1-S710, may participate in the pathology of schizophrenia. By using newly generated a phospho-specific antibody against human DISC1-S713, we observed a significant decrease in this phosphorylation in schizophrenia-derived olfactory immature neuronal cells compared with those from normal controls. To validate this deficit associating with the molecular switch, we examined iPSC-derived neuronal precursor cells and neurons and compared them between schizophrenia and controls. Our strategy will provide important clues for understanding neurodevelopmental process associated with schizophrenia.

Poster Board Number: T-3211

MODELING A HUMAN GENETIC DISORDER OF THE AUTONOMIC NERVOUS SYSTEM IN IPS CELLS

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Congenital Central Hypoventilation Syndrome (CCHS) is a rare disorder of respiratory control that shows significant neural crest (NC)-pathology, affecting cranial, cardiac, and trunk NC cell lineages. CCHS is highly correlated with mutations in the PHOX2B, a paired-like homeodomain transcription factor. CCHS-associated PHOX2B mutations show preservation of the DNA homeodomain structure, and therefore may continue to bind DNA; however, how this interaction affects gene regulation and ultimately cellular function remains unknown. Interestingly, PHOX2B mutations also cause a NC tumor (Neuroblastonoma), and it is unclear how this tumor would be related to CCHS. We have successfully generated induced pluripotent stem (iPS) cells derived from a CCHS autopsy subject and can differentiate these cells into NC cells. CCHS-derived NC cells show a hyper-proliferative and abnormal migratory phenotype. Currently, we do not know which NC cell subpopulation contributes to the hyper-proliferative and/or migratory phenotype, since unique gene signatures of NC subpopulations remain unknown. To identify the unique gene signatures of NC subpopulations we are performing RNA-seq from genetically marked NC cells in mouse embryos, as well as in human iPS-derived NC cells using several different NC differentiation methods. With unique gene signatures, we can then determine the contribution of each NC cell subpopulation in CCHS and hopefully improve regenerative therapies and tissue repair with implications for multiple NC diseases.

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Poster Board Number: T-3212

EFFICIENT AND STABLE EXPRESSION OF NON-VIRAL VECTORS IN HUMAN IPS CELLS: TOWARDS A GENE THERAPY APPROACH FOR HEMOPHILIA.

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Hemophilia A is a congenital bleeding disorder caused by a mutation in the coagulation Factor VIII (FVIII). Successful secretion of FVIII protein from engineered cells within a patient's body could circumvent the current replacement therapies that require frequent infusions of FVIII concentrates. For such cell therapy, induced pluripotent stem (iPS) cells derived from the patient or an HLA-matched donor are an attractive cell source because of enormous cell growth potential, pluripotency, and low immunogeneicity. However, the large size of the FVIII gene prohibits efficient delivery by conventional viral vectors, and strong silencing activity in pluripotent stem cells hinders long-term, stable expression of transgenes. To overcome these issues, we took advantage of the piggyBac transposon vector system. During the evaluation of piggyBac vectors in iPS cells, we noted a loss of reporter expression after long-term (2~3 month) of culture, presumably due to transgene silencing. To stabilize transgene expression, we incorporated a human insulator element, called D4Z4, into our piggyBac vector. Unexpectedly, full-length D4Z4 inhibited the transposition of the vector in target cells. However, 5′ or 3′ fragments of D4Z4 permitted transposition as efficiently as the native vector. After more than 5 months of culture, we show that either the 5′ or 3′ fragment of D4Z4 insulator is sufficient to maintain stable expression in human iPS cells. In addition, similar anti-silencing activity was also observed in *de novo* DNA methyltransferase-null ES cells, suggesting that the activity of the insulator is DNA methylation independent. Next, we cloned a 4.4 kb B domain-deleted FVIII cDNA, as well as 7.0 kb full-length FVIII cDNA into our piggyBac vectors. Even though the transposition rate tends to be lower with longer transgenes, we confirmed expression of the transgene and blood coagulation activity in the culture media of transgenic iPS cells. Lastly, we injected our piggyBac vectors into hemophilia A mice via tail vain by hydrodynamic injection, and successfully detected stable expression of human FVIII protein in plasma for several months without developing the anti-FVIII antibodies. We expect our engineered vectors will facilitate long-term expression of therapeutic transgenes in human pluripotent stem cells and their derivatives.

Poster Board Number: T-3213

SCREENING HUMAN INDUCED PLURIPOTENT STEM CELL DERIVED NEURONS FOR HISTONE DEACETYLASE INHIBITION

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The vast majority of high throughput *in vitro* model systems in neuroscience drug discovery rely on target over expression in non-neuronal cell lines or lower throughput rodent embryonic primary neuronal cultures as human neurons have been difficult to ethically obtain in a reliable and consistent process. The stem cell field has made great strides filling this gap with the discovery of induced pluripotent stem (iPS) cells and protocols for differentiating brain region specific neurons. However, just like with primary cells there are questions concerning variability during differentiation and donor differences to consider especially when looking at drug targets with potential splice variants or subunit heterogeneity. For the application of a differentiated cell model to drug discovery it is critical to have a robust and reliable process of differentiation that will allow the comparison of results over the several years of a discovery project. In this study we characterized Neurons differentiated from human iPS cells in order to understand the application of these cells as a physiologically relevant model system for neuroscience drug discovery. We have developed a high content imaging assay to study the role of epigenetics on synaptic plasticity by measuring changes in the acetylation of histone 4 lysine 12 as well as simultaneously monitoring effects on neurite outgrowth and branching.

Poster Board Number: T-3214

MODELING HUMAN MYOTUBULAR MYOPATHY VIA INDUCED PLURIPOTENT STEM CELLS DERIVED FROM AUTOPSY SKELETAL MUSCLE TISSUE

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INTRODUCTION: Congenital muscular dystrophies (CMDs) are a group of genetic disorders that affect children at birth and clinically manifest as a "floppy" infant lacking muscular tone. In this study, we focus on X-linked myotubular myopathy (XLMTM), a severe form of CMDs. Although the deletion or point mutation of the MTM1 gene has been implicated in the etiology of this disorder, to date its pathogenesis is poorly understood. Recent progress in our understanding of human induced pluripotent stem cells (iPSCs) opened new avenues for modeling human diseases. This dynamic recapitulation of a disease process will help gain insight into disease pathogenesis and might potentially aid in finding specific therapeutic interventions such as gene-based therapy. In this study we generate iPSCs from somatic cells of a patient with XLMTM, which, to our knowledge, has not been demonstrated thus far. In addition, we show differentiation of IPSCs into myocytes and their functional integration. METHODS: Skeletal muscle tissue was collected during the autopsy of a patient with XLMTM and an age-matched healthy individual. The collected muscle tissue was dissected and cultured for three weeks with the goal to isolate fibroblasts. These fibroblasts were transformed with a polycistronic lentiviral vector construct expressing human OCT4, SOX2, KLF4 and cMyc/mCherry. Formed colonies were characterized by immunofluorescent stains, RT-PCR, karyotyping, and microarray studies. Generated iPSCs are injected into SCID mice to demonstrate teratoma formation. Cells were also grown in culture for differentiation into muscle cell lineages and injected *in vivo* to test their potential for differentiation and integration. RESULTS: After two weeks of transduction, a large number of colonies had formed. The colonies show positive immunostaining of Oct4, Tra1-60, and Nanog, as well as expression of human pluripotency genes hSox2, hNanog, hOct4, and hDNMT3B as revealed by RT-PCR. These results support the pluripotent nature of the cells. In addition, teratomas composed of all three germ layers formed when iPSCs were injected into SCID mice. The iPSCs also demonstrate ability to differentiate into cells expressing muscle markers in vitro. CONCLUSIONS: Our study has demonstrated the successful generation of iPSCs from autopsy tissue of a patient with XLMTM--a new tissue source for generating human iPSCs. These patient-specific iPSCs possess the ability to differentiate in vitro and *in vivo*, providing an effective model for myopathy studies.

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Poster Board Number: T-3215

MESENCHYMAL PROGENITORS DERIVED FROM HUMAN INDUCED PLURIPOTENT STEM CELLS

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Introduction: Mesenchymal stem cells (MSCs) have a high potential for therapeutic efficacy in treating diverse musculoskeletal injuries and cardiovascular diseases, and ameliorating the severity of graft-versus-host and autoimmune diseases. However, most of these clinical applications require substantial quantities of cells, but the number of MSCs that can be obtained from a single donor is limited. Moreover, cultured MSCs gradually lose their potency, and eventually cease proliferation following extensive cultivation. Reports on the derivation of mesenchymal progenitors from human embryonic stem cells (ESCs), and more recently also from human induced pluripotent stem cells (iPSCs), are thus most intriguing, since the infinite proliferative capacity of ESCs opens the possibility to generate large amounts of highly uniform batches of MSCs. Materials & Methods: In the present study we derived mesenchymal progenitor cells (iMPs) using three different protocols: (1) embryoid body formation, (2) indirect co-culture with MSCs, or (3) forced differentiation of colonies. Results: Starting from an existing human iPS cell line and a newly established cell line reprogrammed from human MSCs, respectively, we derived iMPs exhibiting typical MSC/fibroblastic morphology. These cells could be expanded more than 20 passages (over 35 population doublings) before reaching senescence and were able to differentiate *in vitro* along the osteogenic (Alizarin Red staining, alkaline phosphatase activity), chondrogenic (GAG assay), and adipogenic (Oil Red-O staining) lineages. Discussion: Until now, there are currently no standard protocols for the derivation of mesenchymal progenitors from pluripotent cells and verification of their mesenchymal nature. Our system of using iPS cells reprogrammed from human MSCs allows us to compare key characteristics of iMPs with the original MSCs from the same donor and draw more specific conclusions on their similarities and differences than would be possible with iMPs and MSCs from different sources and donors.

Poster Board Number: T-3216

A TRIAL OF *IN VITRO* RECONSTITUTION OF HUMAN SKIN USING TRANSGENE-FREE INDUCED PLURIPOTENT STEM CELLS

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Some defined factors which are important for self-renewal of embryonic stem cells (ESCs) have been shown to reprogram both mouse and human somatic cells into ESCs-like pluripotent cells, named induced pluripotent stem cells (iPSCs). Induction of reprogramming by these defined factors had been mostly carried out by co-infection with retroviral vectors. The main problems of this retrovirus-based method were oncogenicity and mutagenesis. Therefore, transgene integration-free iPSCs have been necessary for their future therapeutic application. In our present study, we tried to generate transgene integration-free human iPSCs (hiPSCs) and to differentiate these hiPSCs into keratinocytes and to reconstitute human skin equivalent *in vitro*. To achieve this, firstly, we used the piggyBac-transposon system to deliver the reprogramming factors. The piggyBac transposon is a moth-derived DNA transposon and has been used for gene delivery and mutagenesis. Among several DNA transposons, piggyBac transposon does not leave 'footprint' mutations upon excision. This means removal of the transposon from the host genome without changing any nucleotide sequences. Using this system, we generated hiPSCs from human dermal fibroblasts. After the removal of piggyBac transposon, these hiPSCs can be transgene integration-free and mutation-free, which are invaluable for therapeutic applications. Next, we tried to differentiate these hiPSCs into keratinocytes and obtained a homogeneous population of cells that showed phenotypic characteristics of basal keratinocytes (K5/K14 positive). Finally, we performed three-dimensional culture of these induced-keratinocytes and could observe a pluristratified epidermis. Our results indicated the great promise of hiPSCs in the dermatological regenerative medicine.

Poster Board Number: T-3217

INVESTIGATION OF THE X CHROMOSOME USING INDUCED PLURIPOTENT STEM CELLS FROM HUMAN X ANEUPLOID MODELS

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The goal of this project is to develop a model system to identify genes located on the human X chromosome, which require two copies in normal early development. Turner Syndrome females have complete or partial loss of their second sex chromosome leading to a 45, X karyotype. Three percent of pregnancies start out with a 45,X karyotype, however 99% of these pregnancies are spontaneously terminated. Turner Syndrome accounts for 1 in 2,000 live births, making it one of the most common human chromosomal abnormalities and the only viable monosomy. Turner females have a wide variety of clinical characteristics, including short stature, premature ovarian failure and subsequent infertility. Triple X syndrome (47,XXX) is on the opposite end of the spectrum, with presence of an additional X chromosome. Triple X syndrome occurs in 1 in 1000 life births. Triple X females may be taller on average and have learning disorders, but these women have normal sexual development. Because of the developmental consequences of monosomy X, we hypothesize that induced pluripotent stem cells (iPSCs) derived from Turner Syndrome females will accurately replicate the haploinsufficiency and X chromosome aberrations observed in the original patient, making them, along with Control and Triple X iPSCs, an ideal model with which to study the role of the X chromosome in early development and pluripotency. To address this hypothesis, one Turner syndrome and two Control fibroblast lines were reprogrammed using two sequential retroviral transductions of the four Yamanaka factors (OCT4, SOX2, KLF4, cMYC). To optimize iPSC line generation, a lentiviral vector carrying all four Yamanaka factors in one single excisable vector was used to reprogram the previous lines, and three additional Turner Fibroblast lines (Fetal, Neonate and Child). We observed increased reprogramming efficiency amongst all lines with the use of Valproic Acid (VPA), a histone deacetylase inhibitor. However, Turner Syndrome Child 1 was only reprogrammable with the addition of VPA. Turner iPSC lines exhibit a 45, X karyotype, while the control iPSC lines maintain a normal 46,XX karyotype. All lines express the key plruipotency markers. All lines developed teratomas with tissues characteristic of all three germ layers when injected into SCID mice. *In vitro* differentiation resulted in beating cardiomyocytes and differentiated cells were positive for cells of the three germ layers. Thus, initial characterization of all iPSC lines did not indicate differences in pluripotency between Turner Syndrome and Control iPSC lines. To further investigate possible differences amongst the X chromosome anuploid iPSCs and Control iPSCs we used fluorescence activated cell sorting

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and the microfluidic technology of the Fluidigm system to analyze gene-expression at the single cell level. Using this method we can accurately analyze the expression of 96 genes for each individual pluripotent cell. The iPSC lines were sorted for two pluripotency cell surface markers (SSEA4 and TRA-1-60), then analyzed for the following sets of genes: Pluripotency, X-inactivation, Pseudoautosomal, Escape from X-inactivation, Germ Cell, and the three germ layers. To gain a whole transcriptome view of expression in the X chromosome anuploid and Control iPSC lines, we performed RNA-Seq. Alterations in gene expression between the X chromosome anuploid iPSC lines may indicate causes of the early lethality in Turner Syndrome females and causes of symptoms in Triple X females.

Poster Board Number: T-3218

DONOR AGE AFFECTS TO GENERATE IPS CELLS FROM HUMAN SKIN FIBROBLASTS

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Human induced pluripotent stem cells (hiPSCs) represent a unique opportunity for the field of regenerative medicine because they offer the prospect of generating unlimited quantities of cells for autologous transplantation, with a potential application for use in treatments for a broad range of disorders. Although somatic cells can be reprogrammed into iPSCs by the expression of certain defined embryonic factors, several factors, including age and health status, have been found to show large variations in the efficiency of hiPSC generation. Reprogramming results in the early activation of the pluripotency marker alkaline phosphatase (ALP). This study examined the relationship between the donor age of human skin fibroblasts and the emergence of ALP-positive colonies by iPSC generation when using an episomal approach. Three skin fibroblast lines from normal Japanese donors of varying ages were used; including the TIG-3S (Fetus), TIG-120 (from a 6-year-old donor) and TIG-108 (from a 40-year-old donor) fibroblast lines. Immunofluorescent staining showed all fibroblast lines to be positive for CD13 and CD90. The cells were cultured at an initial seeding density of 1x104 cells/cm2, and the doubling time (Dt) at 1-3 days was estimated based on their respective growth curves, which showed the TIG-3S fibroblasts to grow faster (22.0 ± 0.2 hours) than the TIG-120 fibroblasts (34.3 ± 0.9 hours) and TIG-108 fibroblasts (38.8 ± 1.7 hours). The saturation density (cells/cm2) declined with age (TIG-3S; 9.68 ± 0.14 x 104, TIG-120; 5.36 ± 0.29 x 104, TIG-108; 3.96 ± 0.39 x 104). The colony formation capacity of TIG-3S fibroblasts was higher than that of TIG-120 fibroblasts and TIG-108 fibroblasts. Episomal vectors with pCXLE-hOCT3/4-shp53-F, pCXLE-hSK and pCXLE-hUL (Okita et al., Nat. Methods, 2011) were electroporated into three skin fibroblast lines and then the lines were cultured with fibroblast growth medium to induce hiPSCs. The transfected cells were reseeded onto SNL feeder cells after 6 days, and thereafter were maintained in an embryonic stem cell medium. ALP-positive colonies were detected at 20 days after electroporation. The emergence of ALP-positive colonies decreased in the TIG-120 fibroblasts and TIG-108 fibroblasts in comparison to the TIG-3S fibroblasts. Therefore, iPSC generation from human skin fibroblasts was found to be influenced by the age of the donor somatic cells. The donor age of somatic cells is therefore considered to be a pivotal factor in iPSC generation.

Poster Board Number: T-3219

CONSTRUCTION OF SPECIFIC NONVIRAL VECTOR FOR *EX VIVO* GENE TRANSFERRING AND CORRECTION OF HUMAN B-GLOBIN GENE IN INDUCED PLURIPOTENT STEM CELLS DERIVED FROM A B-THALASSEMIC PATIENT

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The invention of induced pluripotent stem (iPS) cells enables for the first time the derivation of enough numbers of patient-specific stem cells and holds promise for cell-based therapies. Recent studies have revealed the potential of iPS cell generation combined with gene therapy for treatment of different disease in human. In this study, we designed and constructed a nonviral tissue-specific plasmid vector as a gene transfer vehicle to express wild type β-globin gene named pHBB. This vector comprises two main parts: a bacterial backbone for efficient amplification in E. coli cells and a eukaryotic antibiotic resistance ORF, puromycin. Second part contains one expression cassette including β-globin gene enhancers, promoter and complete β-globin gene sequence along with its 3´and 5´ UTRs. Moreover this vector encompasses a specific phiC31 integrase site (attB) which its co-transfection with another vector encoding phiC31integrase enable it to integrate into specific sites named pseudo attP sites in the human genome. On the other hand, two loxP sites flanking of bacterial backbone and puromycin resistant ORF were designed in this vector that facilitate deletion of these sequences by TAT-NLS-Cre recombinase after integration into the genome of target cells. This character reduces possible host immune responses to the vector and potential genotoxicity. The structure of the vector was confirmed through several steps of digestion experiments and sequence analysis. Functional analysis of the plasmid vector was successfully achieved by stable transfection of the vector into genome of a hematopoietic cell line, K562, which is β-globin promoter specific line and able to express β-globin gene. Then keratinocyte derived iPS cells (KiPS cells) are created by using episomal vectors as safe iPS cells with intact genome. The vector used for site-specific recombination (SSR) in pseudo attP sites of iPS cell genome by electroporation. Some of these pseudo attP sites can act as safe harbors for long time acceptable gene expression level. Finally the efficiency of recombinant β-globin production by the vector was determined in hematopoietic cells derived from transected KiPS cells. Thus pHBB can be assumed as an ideal vector for *ex vivo* gene transferring into β-thalassemic patient specific iPS cells to compensate β- globin expression defect by producing 15% or more normal hemoglobin concentrations after hematopoietic differentiation.

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Poster Board Number: T-3220

NEURAL PRECURSORS DERIVED FROM HUMAN IPS CELLS CAN BE USED IN THE TREATMENT OF BRAIN AND SPINAL CORD INJURY

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No treatment currently exists to restore lost neurological function after stroke or spinal cord injury. A growing number of studies have highlighted the potential of stem cell and more differentiated neural cell transplantation as intriguing therapeutic approaches for neural repair after stroke. In this study, we used human induced pluripotent stem cell (IMR90)-derived neural precursors (iPS-NPs) for transplantation in a rat middle cerebral artery occlusion (MCAO) model of stroke or a balloon-induced spinal cord compression lesion (SCI). Neural precursors were derived from iPS through the micro-aggregate stage using 300 ng/ml noggin and 20 uM SB. Prior to *in vivo* experiments, iPS-NPs were differentiated in vitro and the results analyzed. Subsequently, the percentage of cells expressing markers of neural precursors, including oct3/4, sox2, SSEA-4, SSEA-1, TRA-1-60, CD24, CD133, CD56, betaIII-tubulin, NF70, nestin, CD271 and CD29, was assessed by FACS in undifferentiated and predifferentiated (FGF and EGF omitted from the culture medium (CM) for 7 days) NPs. In vitro we were able to differentiate iPS-NPs into GABA-ergic neurons and, with less efficiency, into motor neurons. Female Sprague-Dawley rats were subjected to focal cerebral ischemia by reversible right MCAO for 90 min, while male Wistar rats were used for SCI. A suspension of iPS-NPs (300 000 cells in 3ul of CM) was transplanted into the lesions 7 days after MCAO or SCI (n=14, n=12); the control groups (n=8) were injected with saline. Metabolic profiles in the striatal tissue of both hemispheres were assessed by magnetic resonance spectroscopy (MRS) in the MCAO model. Four months after MCAO, MRS revealed that the concentrations of brain metabolites (glutamate, glutamine, N-acetyl-aspartate, creatine, taurine, choline and inositol) in grafted animals returned nearly to the values found in nonlesioned animals. Functional recovery in rats with an MCAO lesion was regularly assessed after transplantation by the apomorphine-induced rotation test, the tape-removal test or the rotating pole test. Rats with SCI were tested using the BBB (motor) and plantar (sensory) tests. The grafted animals in the stroke model displayed a decreased number of clockwise rotations in the apomorphine rotation-induced test and performed better in the tape removal test. Animals with SCI that received cell transplants significantly improved their BBB score when compared to control animals. iPS-NPs robustly survived in both models of injury, maintained their neural phenotype, migrated toward the lesioned area and survived there for 2-4 months after transplantation. In addition, some of the cells differentiated into more mature and tissue-specific neurons (NSE-, MAP2- and DARPP32-positive cells). No tumor formation was observed throughout the study. In conclusion, these results suggest that iPS-NPs undergo further differentiation after transplantation, integrate into the neural tissue, partially improve functional outcome and can serve as a tool for cell transplantation therapy.

Poster Board Number: T-3221

OPTIMIZING TOPOGRAPHICAL FEATURES OF ELECTROSPUN NANOFIBERS FOR EFFICIENT ENDODERMAL DIFFERENTIATION OF HUMAN INDUCED PLURIPOTENT STEM CELLS

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Human induced pluripotent stem cells (hiPSCs) are embryonic-like stem cells and can be induced to differentiate into three primary germ layers, ectoderm, mesoderm and endoderm. Mass production of functional somatic cells by in-vitro endodermal differentiation of hiPSCs is considerable for curing of diseases related to endoderm-derived organs such as liver and pancreas. Present protocols for endoderm differentiation are limited to optimization of soluble factors (e.g. growth factors) as their concentration, composition and treating time. However, this approach isn't efficient enough. There are various parameters influencing lineage commitment of stem cell, including cell-cell interactions, mechanical forces and cell-extracellular matrix interactions. The latter has been attracted more attention to improve efficiency of differentiation. It has been shown that extracellular matrix (ECM) is comprised of fibers in micro/nano scale. By mimicking ECM topography through engineering of nanofibrous substrates, one can achieve more efficient differentiation. In this work, nanofibrous substrates have been fabricated with different topographies by electrospinning of poly(ε-caprolactone). The effect of topographical features, including fiber diameter, fiber orientation and pore size on endodermal differentiation of hiPSCs has been studied and the optimum topography has been selected. The results indicated that endodermal differentiation of hiPSCs can be influenced siginificantly by the variations in topographical features.

Poster Board Number: T-3222

IN VITRO CHEMOSENSITIVITY OF HUMAN GLIOMA STEM CELLS COMPARED WITH THAT OF NORMAL NEURAL STEM CELLS FROM HUMAN IPS CELLS

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Glioma stem cells (GSCs) were initially identified from glioma tissues as cells that formed spheres *in vitro*, like normal neural stem cells (NSCs). It was later found that GSCs and NSCs have some similar properties. However, their detailed biological properties, especially their chemosensitivities to various reagents, remain to be investigated. In this study, we examined the *in vitro* chemosensitivity of GSCs compared with NSCs prepared from human induced pluripotent stem (iPS) cells (iPS-NSCs), which we used as normal controls. Three GSC lines isolated from surgically removed glioblastoma tissues and cultured as neurospheres for over three months were used. Human iPS cells (clone. 201B7) generated from fibro-

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blasts were differentiated to iPS-NSCs by embryoid body formation or SMAD inhibitors treatment, and propagated as neurospheres. NSCs derived from human fetal neural tissues (fetal-NSCs) were used as an additional normal control. The *in vitro* chemosensitivity to 11 reagents (procarbazine, 6-MP, CDDP, VP-16, gefitinib, vincristine, Ara-C, ACNU, temozolomide, 5-FU, and MTX) was examined using two assays. The number of viable cells was estimated by measuring the total ATP content in the cells, and the number of non-viable cells was determined by the release of lactate dehydrogenase (LDH) from cells with damaged membranes. All three GSC lines, the iPS-NSCs, and the fetal-NSCs showed similar chemosensitivities to procarbazine and gefitinib. However, the GSCs, iPS-NSCs, and fetal-NSCs responded differently to the other reagents. These findings suggest that GSCs and normal NSCs show different chemosensitivities to several regents. Examining these differences will increase our knowledge about the cellular properties of GSCs and provide useful information for the development of new drugs that target GSCs.

Poster Board Number: T-3223

SYSTEMATIC SHRNA-MEDIATED SILENCING OF GENES IMPLICATED IN AUTISM SPECTRUM DISORDERS AND SCHIZOPHRENIA IN IPSC-DERIVED HUMAN NEURONS

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Autism spectrum disorders (ASD) and schizophrenia are highly heritable disorders of human cognition and behavior. Advances in sequencing technology have allowed for better refinement of genetic etiology of complex disorders, and numerous genes and microdeletion/microduplication regions have been associated with both disorders, with some overlap. However, it remains challenging to establish a causal role for genetic variants associated with disease risk. One aim of this study is to investigate important implicated genes in these related disorders by identifying aberrant phenotypes in a genetic knock-down system. This is particularly relevant for elucidating the relative contributions of genes encompassed within microdeletion/microduplication syndromes such as 16p11.2 or 22q11.2, where the presumed causative gene(s) has not been found. To complement mutation analysis studies, we developed and characterized a human neural progenitor cell line derived from induced pluripotent stem cells (iPSC-NPCs) taken from fibroblasts from a psychiatrically screened healthy male. iPSC-NPCs were karyotypically normal, differentiated into all central nervous system cell types assessed, and were capable of synapse formation upon long-term differentiation. We selected over 100 genes associated with ASD and/or schizophrenia and targeted each gene with > four short-hairpin (sh) RNA's/gene, delivered by lentivirus along with a series of control shRNAs not known to target any human gene. In total, over 400 stable cell lines were generated and all cell lines were validated by qPCR in comparison to control shRNAs. A battery of phenotypic assays at both the progenitor and mature neuron stage were developed that assess a range of phenotypes including cell growth, cell extension, and intracellular calcium release. Changes in these cellular phenotypes occur upon knockdown of several of the GWAS-nominated candidate genes suggest that these genes may play a functional role in microdeletion/microduplication syndromes. These results can help the research community prioritize and direct further investigation of the function of genes associated with ASD and schizophrenia.

Poster Board Number: T-3224

EXOGENOUS SIGNALS OPPOSITELY AFFECT ADIPOCYTE DIFFERENTIATION AND NEURAL DIFFERENTIATION OF HUMAN ES/IPS CELLS

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Efficient differentiation of human pluripotent stem cells (hPSCs) into specific cell lineage is required for disease modeling and for regenerative medicine. We have previously reported adipocyte and neural differentiation from human ES cells (hESCs) and iPS cells (hiPSCs). In this study, we found the efficient differentiation of hPSCs into adipocytes by regulating the concentration of knockout serum replacement (KSR) during embryoid body formation, which is commonly used as serum replacement supplement for maintaining hPSCs. Although KSR has been known to contain growth factors which may affect the differentiation of PSCs, its effects on the differentiation of hPSCs into adipocytes and neural lineage are still unknown. Here, we investigated how the concentration of KSR affects adipocyte and neural differentiation of hPSCs (5%~30% in the medium). Considering the differentiation propensity of hPSCs, we used 4 cell lines (2 lines of hESCs and 2 lines of hiPSCs). We evaluated the efficiency of adipocyte differentiation by Oil red O staining and the expression of adipocyte marker genes, PPARγ2 and CEBPα, and examined neural differentiation potential by the expression of neural marker genes, PAX6 and NCAM. We observed high concentrations of KSR induced adipogenesis but inhibited neural differentiation in a concentration-dependent manner, except for one hESCs line, KhES3. KhES3 differentiated into adipocytes efficiently even in low concentrations of KSR, but did not differentiate into neural lineage effectively. Taken together, these results indicate that KSR, basically, negatively regulate neural differentiation but positively regulate adipocyte differentiation. Because KSR contains high concentrations of insulin (about 6.7μg/ml at 10%KSR in medium), it may have triggered a series of transcription factors regulating adipogenesis and then may have promoted the adipocyte differentiation.

Poster Board Number: T-3225

TROPHOBLAST DIFFERENTIATION FROM HUMAN INDUCED PLURIPOTENT STEM CELLS TREATED WITH BMP4

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Models appropriate for studying human trophoblast development are limited, especially ones to study transition between the undifferentiated cell and early trophoblast stages. Treating human embryonic stem cells (ESC) with the growth factor, BMP4 (termed hESC/BMP4), is a powerful means for generating trophoblast and provides a series of temporal and spatial snapshots of the changes that accompany initial differentiation from precursor pluripotent cells to the subsequent formation of sublineages as they arise from cytotrophoblast precursors. Attempts to improve differentiation to trophoblast can be achieved by blocking the signaling systems essential for maintaining pluripotency such as activin A and FGF signaling simultaneously. This involves exposure to A83-01 (an inhibitor of activin A signaling superior in both specificity and potency to SB431542) and PD173074, a fibroblast growth factor receptor inhibitor. The hESC/BMP4 system improves our understanding of the mechanisms that underpin the initial emergence

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of trophoblast from its stem cell precursors as well as when and how the various trophoblast sub-lineages differentiate. If the hESC/BMP4 system can be applied to induced pluripotent stem cells (iPSC), it opens ways to study connections between genetic/epigenetic background and pregnancy disorders associated with trophoblast phenotype that have been difficult to address until now. In other words, it might allow a recapitulation of features of a prior placental phenotype after a baby is born. In our work, iPSC are routinely established by either retroviral or lentiviral transduction of reprogramming factors into somatic cells. However, as continued expression of the transgenes may interfere with downstream differentiation processes, alternative methods have been used to generate integration-free iPSC. Here we have developed a dozen lines of human iPSC from mesenchyme outgrowths of umbilical cords collected at birth either by integrating virus carrying tetracycline-inducible lentiviral vectors or by an episomal plasmid-based approach initially developed in the Yamanaka's laboratory to establish integration-free iPSC. Four iPSC lines were established from umbilical cord mesenchyme infected with virus carrying the OCT4/SOX2/KLF4/cMYC/NANOG/LIN28 reprogramming genes at ~0.1% efficiency. Eight other primary cultures were transfected with episomal plasmids, which reprogrammed cells with about one-tenth the efficiency of retroviruses. Three of the former and one of the latter cell lines were tested for differentiation towards trophoblast in response to addition of BMP4 and the activin A/FGF inhibitors. The cell lines produced CG, P4, and PGF, indicators of advanced trophoblast sub-lineages, as efficiently as H1 and H9 hESC, with no evidence for generation of mesoendoderm derivatives. Our data demonstrate that iPSC derived from umbilical cords can be converted efficiently and unidirectionally to trophoblast. This approach will allow us to examine features of sub-lineage differentiation, including invasiveness, hormone production, oxygen sensitivity, and response to various stressors implicated in placental diseases such as preeclampsia.

Poster Board Number: T-3226

APPLICATION OF DRUG-CONTROLLABLE GENETIC SWITCH FOR GENERATION OF HUMAN IPS CELLS.

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Human induced pluripotent stem cells (iPS) are generated by reprogramming of somatic cells through enforced expression of embryonic transcription factors. However, clinical applications require that expression of introduced transgenes must be permanently switched off in the iPS cells and obtained differentiated progenies. Here, we took advantage of epigenetic switch that relies on doxycycline(dox)-controllable binding of tTRKRAB transrepressor to tetO element. In the presence of dox tTRKRAB is sequestrated from tetO allowing for transgenes expression. In contrary, dox removal allows for tTRKRAB binding to tetO that results in tight transcriptional repression of proximal promoter through heterochromatin formation. In order to apply this system for reprogramming, the tetO element was inserted into pSTEMCCA lentiviral vector carrying OCT4, SOX2, KLF4 and cMYC under control of EF-1alpha promoter. Co-transduction of human skin fibroblasts with obtained pSTEMCCA-tet and pLV-HK carrying tTRKRAB cDNA followed by culture in presence of doxycycline allowed for expression of reprogramming factors and thus efficient generation of human iPS clones. Obtained clones were picked and further cultured in the absence of dox. Tight repression of introduced transgenes in all human iPS clones was analysed by RT-qPCR and confirmed full functionality of our system. Pluripotent phenotype of iPS cells was revealed by analysis of endogenous embryonic genes expression using RT-PCR and cell surface protein markers by immunofluorescence. tTRKRAB-mediated epigenetic repression persisted through prolonged culture of obtained iPS cell lines. Importantly, expression of introduced transgenes remained undetectable after differentiation into embryonic bodies. Our results confirm that our epigenetic switch effectively prohibits re-expression of embryonic transgenes in human iPS cells and their differentiated progenies paving the way for their applications in various fields of regenerative medicine, disease modelling and drug discovery.

Poster Board Number: T-3227

PERICELLULAR MATRIX OF DECIDUA-DERIVED MESENCHYMAL CELLS IS A VERSATILE HUMAN SUBSTRATE FOR BOTH FEEDER-CELL-FREE GENERATION AND LONG-TERM MAINTENANCE OF HUMAN-INDUCED PLURIPOTENT STEM CELLS

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Human-induced pluripotent stem cells (hiPSCs) have been generated from various somatic cells by inducing defined sets of transcription factors, and the properties of these hiPSCs are similar to those of human embryonic stem cells (hESCs). Generally, the use of hiPSCs should make great contributions to the field of regenerative medicine and to our understanding of the molecular pathogenesis underlying many intractable diseases. The clinical promise of cell-based therapies using hiPSCs has driven an intense search for ideal cell sources, reprogramming methods, and cell culture systems. However, the details remain to be investigated. hiPSCs/hESCs are generated and maintained on living feeder cells, such as mouse embryonic fibroblasts or SNL cells, or on feeder-free culture substrates with Matrigel, fibronectin, vitronectin, or human recombinant laminin-511. Clinical applications require quality-controlled xenobiotic-free culture systems to minimize health risks from animal-derived pathogens and immunogens. Therefore, the use of human-derived primary living cells, such as human fibroblasts or amnion-derived cells, is a hopeful approach, although there are difficulties to overcome. Previously, we reported that the pericellular matrix of decidua-derived mesenchymal cells (PCM-DM) is an ideal human-derived material for maintaining hiPSCs/hESCs. The activity of PCM-DM in maintenance of hiPSCs/hESCs is similar to that of Matrigel and the preparation of a PCM-DM is easy and reproducible, as decidua-derived mesenchymal cells (DMCs) can be prepared and expanded in large quantities. Moreover, we reported success generating hiPSCs from DMCs on PCM-DM via the retroviral transduction of four factors: OCT4, SOX2, KLF4, and c-MYC. The established hiPSCs had stable cellular properties, such as alkaline phosphatase (ALP) activity, hESC-specific genes and surface markers, and differentiation ability in vitro and *in vivo* for long culture periods, when early and later passages were compared. This study

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conducted a global gene expression analysis of hiPSCs cultured on PCM-DM at early and later passages in order to estimate their cellular properties in detail. We found that the expression levels of GDF3, FGF4, UTF1, and XIST varied with culture time, although the expression of these genes did not affect pluripotency. Moreover, cluster analysis showed that the differences in gene expression patterns between clones were less than those between early and later passages for each clone, and established hiPSCs tended to resemble control hiPSCs and hESCs cultured on feeder cells in terms of gene expression patterns. We also confirmed copy number variation (CNV) at early and later passages. There was no correlation between CNV and gene expression patterns, although some CNV involved genes that are often reported in hiPSCs/ hESCs cultured for long periods. These findings suggest that PCM-DM is a practical, human-derived substrate that can be used not only for the stable maintenance of hiPSCs, but also for their generation.

Poster Board Number: T-3228

VALIDATION OF *IN VITRO* METHODS FOR DETECTION OF RESIDUAL UNDIFFERENTIATED CELLS IN RETINAL PIGMENT EPITHELIAL CELLS DERIVED FROM HUMAN INDUCED PLURIPOTENT STEM CELLS

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Human pluripotent stem cells (hPSCs) have the ability to differentiate into a variety of cells and to self-renew in vitro. Because of these two characteristics, it has been expected that they would provide new regenerative medicine/cell therapy. Recently, it has been getting big attention that clinical trials have been conducted with retinal pigment epithelial (RPE) cells derived from human embryonic stem cells (hESCs) to treat patients with dry age-related macular degeneration and Stargardt's macular dystrophy in the US. For the clinical use of products derived from hPSCs, it is essential to improve both the efficacy and safety of the final product. In order to develop safe hPSC-based treatments, the hurdle of tumorigenicity arising from undifferentiated cells must be overcome. To address the issue of tumorigenicity, some recent publications have advocated the development of protocols for the derivation of hPSC and have outlined methods for the elimination of residual hPSCs. However, to date, there are no capable methods for detection of a small number of residual undifferentiated cells. To locate residual undifferentiated cells, *in vivo* teratoma formation assays have been performed with immunodeficient animals, but more than several hundred cells are necessary for human pluripotent stem cells to form a tumor in immunocompromised mice. Therefore, highly sensitive tumorigenicity assays and their standardization are necessary for detecting a small population of residual undifferentiated cells in products derived from hPSCs. To solve this issue, we evaluated three methods for detection of residual undifferentiated human induced pluripotent stem cells (hiPSCs) in hiPSCs-derived RPE cells: soft agar colony formation assay, flow cytometry and qRT-PCR. These assays were used on cell mixtures that contained defined numbers of undifferentiated hiPSCs in primary RPE cells. Although the soft agar colony formation assay unsuitable for detecting residual undifferentiated hiPSCs, the flow cytometry assay using anti-TRA-1-60 antibody detected 0.1% undifferentiated hiPSCs that were spiked in primary RPE cells. Moreover, qRT-PCR with a specific probe and primers targeting Lin28 transcripts can detect at least 0.01% contamination of residual undifferentiated hiPSCs in primary RPE cells. Based on these results, we examined whether qRT-PCR for Lin28 was applicable in the detection of residual undifferentiated cells in differentiated RPE cells from hiPSCs. As a result, RT-PCR using probes and primers targeting Lin28 transcripts can detect levels as low as 0.002% residual undifferentiated cells in hiPSC-derived RPE cells, namely, that a single hiPSC in 50,000 RPE cells is detectable. We expect our findings to contribute to the process of validation and quality control of hiPSCs-based cell therapy products and to promote the application of regenerative medicine in the treatment of a wide variety of diseases in the near future.

Poster Board Number: T-3229

GENERATION OF INTEGRATION-FREE HUMAN DENTAL-PULP-DERIVED IPS CELLS FROM HLA-HOMOZYGOUS DONORS USING SENDAI VIRUS RNA VECTORS.

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[Abstract] Efficient and integration-free methods to generate human iPS cells are desirable for the future application of regenerative medicine with decreasing the risk of tumorigenesis. Here we show that Sendai virus (SeV) vectors, non-integrating and cytoplasmic-replicating RNA virus vectors, efficiently generated integration-free human dental-pulp-derived iPS cells (DP-iPSCs). We have established multiple DP-iPSC lines from two putative human leukocyte antigen (HLA)-homozygous donor cells (DP-74 and DP-94) that match ~20% of the Japanese population at major HLA loci. We used SeV vectors carrying four reprogramming factors independently, OCT3/4, SOX2, KLF4 and c-MYC/L-MYC, or two sets of three-in-one vector in different order of OCT3/4, SOX2 and KLF4 genes (KOS or OSK), with or without MYC. The highest induction efficiency was achieved when using three-in-one vector (KOS) with c-MYC. However, the phenotypes of obtained iPS colonies were similar: all the colonies tested were transgene-free and showed human ES markers and pluripotency. We conclude that SeV vectors may provide human iPSCs suitable for autologous and allologous stem-cell therapy in the future. [Grant acknowledgement] This work was in part supported by JST, PRESTO.

Poster Board Number: T-3230

NOVEL REAL-TIME PCR-BASED DNA DAMAGE QUANTIFICATION METHOD REVEALS HUMAN PLURIPOTENT STEM CELLS TO BE SELECTIVELY PROTECTED AGAINST ROS-INDUCED DNA DAMAGE

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In this study, we present a method for real-time PCR-based quanitification of DNA damage in human stem cells (long-run DNA damage quanitification, LORD-Q). The LORD-Q method enables highly accurate, time-saving, non-radioactive, high througput DNA damage determination. Human induced pluripotent stem cells (hiPS cells) represent a promising tool for regenerative medicine, circumventing embryonic stem (ES) cell research-associated ethical issues and ES cell-mediated xenograft complications. Tissues derived from patient-specific iPS cells open up new avenues for the development of therapies for various diseases and provide a valuable tool for use in embryo-toxicity assays. Pluripotent stem cells have been described to be highly vulnerable to DNA damaging

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agents, such as ionizing radiation (IR) or alkylating chemicals. Applying the LORD-Q method, we demonstrate that human iPS cells are selectively less vulnerable to DNA damaging agents or radiation compared to their parental somatic cells. At sublethal dosages, hiPS cells exhibit a higher repair capacity of genomic and mitochondrial DNA damage than human fibroblasts. Furthermore, iPS cells are resistant to high dosages of UV radiation and reactive oxygen species. In contrast, treatment with low dosage double-strand break-inducing agents lead to substantial DNA damage initiated apoptosis. Taken together, we demonstrate that our newly developed method is a sensitive tool to monitor DNA damage repair kinetics. We found that hiPS cells are protected against high dosages of ROS and UV radiation, but are vulnerable to DNA double-strand break-inducing agents. In addition, LORD-Q allows to discriminate between DNA damage and other cytotoxic effects caused by harmful agents or radiation. Future applications of this method may reveal the mechanisms protecting or sensitizing stem cell DNA, respectively.

Poster Board Number: T-3231

THE ORIGIN OF SOMATIC MUTATIONS IN HUMAN INDUCED PLURIPOTENT STEM CELLS

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Defined transcription factors can induce epigenetic reprogramming of adult mammalian cells into induced pluripotent stem cells (iPSCs). Recent studies have revealed that iPSC lines acquire fixed point mutations enriched in cancer-related genes immediately after the process of reprogramming. However, the source of these reprogramming-associated mutations remains unclear. It is unknown if many reprogramming-associated mutations occur during the reprogramming process or are inherited from rare mutations in the progenitor cell population. Here we characterize in detail the origin of reprogramming-associated mutations using novel sequencing techniques. We demonstrate that the iPSC and embryonic stem cell (ESC) genomes are relatively stable and uniform at the point mutation level during short-term culture, and that subcloning does not inherently introduce mutations. We also demonstrate a novel analysis technique that allows enhanced high-throughput detection of low-level progenitor cell mutations that have become fixed in iPSC lines. With these results, we are able to separate an elevated mutation rate during reprogramming from inheritance of rare progenitor mutations. Our findings will lead to a greater understanding of the origin of reprogramming-associated point mutations, and will aid in the development of safe iPSCs for clinical use.

Poster Board Number: T-3232

SITE SPECIFIC GENOME EDITING OF HUMAN INDUCED PLURIPOTENT STEM CELLS BY PNA

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Targeted genome editing of human induced pluripotent stem cells (iPSCs) is a useful tool in utilizing the potential of iPSC technology. Nuclease based genome editing technologies, such as Zinc-finger Nucleases (ZFNs) or TAL Effector Nucleases (TALENs), are demonstrated to be effective and efficient in human iPSCs. However, complexity of the enzyme design and high production cost hinder the routine use of these technologies. Here, we utilize an artificial DNA to define a digestion site to engineer a specific locus of the genome in human iPSCs. In this system, peptide nucleic acid (PNA) is used to invade into the target sequence of double strand DNA for exposing single-strand DNA (ssDNA) regions in both sides. Exposed ssDNA regions are digested by a ssDNA specific nuclease or chemical ssDNA cutter Ce(IV)/EDTA complex to induce double strand break (DSB). We confirmed the activity and specificity of this system by digesting a plasmid DNA *in vitro*. Next, we optimized the transfection condition of Cy3-labbeled PNA molecules in human iPSCs by electroporation. PNAs were efficiently introduced into nuclei assessed by confocal microscopy. No severe cytotoxicity of Ce(IV)/EDTA complex was observed under working concentrations. Currently, we have been investigating the digestion activity and specificity of the system using the extracted genomic DNA. We expect this system might offer an alternative tool for editing human genome of stem cells, such as introducing a point mutation or facilitating homologous recombination.

Poster Board Number: T-3233

IN VITRO NEPHROGENESIS OF HUMAN INDUCED PLURIPOTENT STEM CELLS

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In vitro differentiationof human induced pluripotent stem cells (hiPSC) towards the renal lineage might require careful direction by providing cues in the culture medium for the activation of transcription factors related to the development of the nephros.The selection of these factors is provided by the current knowledge of organogenesis of the kidney. Attempts to induce mouse pluripotent stem cells to mature kidney cell types by applying growth factors like BMP, activin A or retinoic acid have been partially successful in deriving cells of the renal lineage, but their specific developmental identity with respect to nephrogenesis could not be deduced . Also, *in vitro* organ cultures from embryonic rudiments have demonstrated the necessity of multiple signals from the environment, usually accomplished only by usage of conditioned media from ureteric bud or metanephric mesenchyme cultures or by direct co-cultures. Thus, the exact combination and timely order of many morphogens and extracellular matrix components to direct renal differentiation *in vitro* has to be determined. The aim of our study is to screen growth factors and matrix signals to develop a renal differentiation protocol. Our methodology utilizes high content screening (HCS) to examine the effect of different concentrations, combinations and sequential addition of molecular environments on iPSC differentiation.As a tool for screening, we generated a hiPSC line that is stably transfected with a transposon containing an eGFP gene driven by the brachyury promoter as an indicator for the mesodermal differentiation and a histone (H2B)-mCherry marker that is utilized in image analysis for cell identification. Using this approach, we successfully identified conditions for the derivation of mesodermal cells that are positive for markers typical for intermediate mesoderm. Screens for further differentiation of these intermediate mesoderm cells along the renal lineage to generate cells that can be used for *in vitro* applications like drug screening or cell based therapies will be presented.

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Poster Board Number: T-3234

MODELLING NEUROGENESIS IMPAIRMENT IN DOWN SYNDROME DISEASE USING INDUCED PLURIPOTENT STEM CELLS DERIVED FROM HUMAN MONOZYGOTIC TWINS DISCORDANT FOR TRISOMY 21

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Down syndrome (DS), caused by a trisomy of chromosome 21, is the most common chromosomal disorder, having an incidence of 1 in 800 live births. It results in varying degree of physical and mental retardation. At present, even though a few genes and anomalies are known, the pathogenic mechanisms underlying the abnormalities remain obscure. The defects described in DS are thought to be closely associated with a group of genes mapped to a specific region of human chromosome 21q22 called Down syndrome critical region (DSCR). Here, we report the generation and the characterization of induced pluripotent stem cells (iPSCs) from fibroblasts obtained from human monozygotic twins discordant for trisomy 21: Twin-N-iPSCs for the normal and Twin-DS-iPSCs for the DS-affected iPSCs. To document their development potential *in vivo*, iPS cells were injected intramuscularly into immunodeficient SCID mice. *In vitro*, iPS cell lines were also characterized to confirm their potential to differentiate into neural progenitor cells (NPCs, day 21 of neuronal differentiation) and mature neurons (day 40 of neuronal differentiation). The derived iPSCs were evaluated to confirm the disease-specific genotype of their parental somatic cells. As revealed by karyotype and high-resolution array-based comparative genomic hybridization (aCGH) analysis, contrary to Twin-N-iPSCs, Twin-DS-iPSCs showed the characteristic trisomy 21. Neuronal differentiation of Twin-DS-iPSCs *in vivo* revealed an abnormal teratoma formation in NOD-SCID mice. Moreover, *in vitro*, Twin-DS-iPSC-derived neurospheres showed a reduced number of NPCs by comparison with the normal ones. This effect was associated with a decrease of cell proliferation and an increase of cell death. When NPCs were further induced to maturate into neurons, we found structural changes in the architecture and density of neurons, glial cells and oligodendrocytes populations together with alterations in the expression of genes involved in lineage specification in neurogenesis and brain development. Furthermore, we provided new insights into the molecular mechanisms underlying these defects. In conclusion, these findings establish iPSCs generated from human monozygotic twins discordant for trisomy 21 as a unique model to recapitulate DS phenotype *in vitro*, study the detailed mechanisms involved in the pathogenesis of DS and design new therapies.

Poster Board Number: T-3235

GENERATION AND CHARACTERIZATION OF RETINAL PIGMENT EPITHELIAL (RPE) CELLS DERIVED FROM HUMAN EMBRYONIC STEM (ES) AND INDUCED PLURIPOTENT STEM (IPS) CELLS WITH DIFFERENT AGE-RELATED MACULAR DEGENERATION (AMD) RISK ALLELES

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Age-related macular degeneration (AMD) is the leading cause of severe vision loss in industrialized nations. The dry form of AMD involves initial damage of the retinal pigment epithelial (RPE) cells, followed by the irreversible loss of retinal photoreceptor cells. Among the multiple genetic loci that have been reported to associate with AMD, certain haplotypes in the complement factor H (CFH) gene have been implicated in a significant increased risk for dry AMD. To investigate the disease mechanisms of AMD and to establish *in vitro* models for this disease, we have generated induced pluripotent stem (iPS) cells from human subjects with different CFH haplotypes. Primary skin fibroblasts obtained from healthy volunteers and AMD patients and fetal RPE cells were transduced with lentiviral vectors expressing the Yamanaka reprogramming factors. The resulting iPS cells were further passaged and characterized using stem cell markers. These iPS cells and human H9 embryonic stem (ES) cells as a control were differentiated in bFGF-free ES cell medium to obtain RPE cells. Molecular analysis of RPE specific gene expression and RPE functional assays including the visual cycle and phagocytosis were performed. The human ES and iPS cell-derived RPE cells with different genetic backgrounds showed variations in gene expression and functional characteristics. These results suggest that human ES and iPS cell-derived RPE may serve as useful models to study AMD and repair damaged retina in patients.

Poster Board Number: T-3236

COMPARATIVE DEVELOPMENTAL GENOMICS OF THE LIVER IN HUMANS AND CHIMPANZEES

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Induced pluripotent stem cells (iPSCs) are potentially powerful tools for comparative studies on cellular differentiation and development. We have successfully generated a panel of iPSC lines from healthy humans and chimpanzees using retroviral transfection with hOCT4/POU5F1, hSOX2, hKLF4, hMYCL1 and hNANOG. We have confirmed that these human and chimpanzee iPSC lines, which express pluripotency-associated markers, can differentiate into all germline tissue layers in embryoid body formation assays. Previous work has indicated that gene expression patterns in the livers of these two species differ as a consequence of evolutionary pressures following the chimpanzee-human split. We have therefore employed staged protocols to differentiate the human and chimpanzee iPSCs into hepatocyte-like cells, and have quantified gene expression differences during this differentiation time course. Our approach, which combines the developmental potential and flexibility of iPSCs with evolutionary biology, has generated an

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unprecedented resource with which to carry out comparative developmental assays in humans and other species.

Poster Board Number: T-3237

ALS-RELATED GENE EXPRESSION CHANGES IN DEFINED POPULATIONS OF HUMAN IPS-DERIVED MOTOR NEURONS

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Amyotrophic lateral sclerosis (ALS) is a uniformly fatal disease of adults characterized by degeneration and death of motor neurons in the spinal cord. The motor neurons most severely affected are those that innervate the distal limb muscles, which derive during embryogenesis from the lateral motor column (LMC) at brachial and lumbar levels. There is much data to suggest that motor neuron degeneration in ALS results in part from a toxic cellular environment. Nevertheless, there is clear evidence in mouse models for determinants of disease onset that are motor neuron-intrinsic. We are using expression data from human motor neurons in vitro and *in vivo* to identify gene expression differences between ALS and controls, focusing on the vulnerable limb-innervating populations. Cultures of motor neurons from human ESC/iPSC lines typically contain a majority of other cell types, a serious confound for gene expression studies. The only protocols for human motor neuron purification to date involve stable Hb9::GFP reporters, which are not feasible to generate for large collections of iPS lines. We therefore developed a method for motor neuron enrichment that is applicable to any hiPSC line. Following infection with a lentiviral vector expressing Hb9::RFP (a gift from C. Marchetto and F. Gage), mixed cultures were allowed to mature and then FACS sorted. The resulting populations contained ~70% of neurons positive for the marker ISL1. Enriched motor neurons from 4 ALS lines with SOD1 mutations and 2 controls were profiled on Affymetrix microarrays. These motor neurons were immature and had not been exposed to ALS-related stressors so, as expected, many genes showed little change. However, a subset of genes showed strong differences in expression levels between ALS and controls. Many of these genes are related to ALS-relevant properties such as hyperexcitability, excitotoxicity and specificity for limb innervation. This suggests that motor neuron-intrinsic differences related to ALS appear early during maturation and therefore may be accessible to study in culture. In order to study the influence of these and other pathways on ALS-related phenotypes in culture, it will likely be necessary to focus on the most vulnerable, limb-innervating, motor neurons. Using the Hox cofactor FoxP1 as a marker for LMC motor neurons we have developed a protocol for enrichment of such cells using early neuralization and modulation of hedgehog signaling. This combination of small molecules generates cultures containing up to 50% of human motor neurons in only 3 weeks. Of these, >70% were limb-innervating motor neurons of the LMCm division, which innervates flexor muscles of the distal limb. These will be a valuable tool for many studies of human motor neuron phenotypes in ALS. This work was funded by Project A.L.S., P2ALS, NYSTEM, Helmsley Foundation and NINDS (NRSA fellowship to DHO).

Poster Board Number: T-3238

MITOCHONDRIAL DNA PROFILES OF PRIMATE SOMATIC AND IPS CELLS.

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Aging and age-related diseases are generally associated with mitochondrial dysfunctions. We determined if skin fibroblasts derived from adult and aged rhesus macaques accumulate mtDNA mutations. We also studied pluripotent stem cells derived from these fibroblasts using induced pluripotent stem (iPS) cell or somatic cell nuclear transfer (SCNT) approaches. IVF-derived ESCs isolated from the same females that provided their skin cells were used as controls. We utilized a massive parallel mtDNA sequencing approach to determine mutations and heteroplasmy. The results demonstrated that both ESCs and SCNT-ESCs, representing the germ line (oocyte) mtDNA, contain a significant number of heteroplasmic variants (852-1685) in addition to the main mtDNA haplotype. However, the frequency of these heteroplasmic variants was relatively low (4.6-6.5%). As expected, adult skin fibroblasts (somatic mtDNA) from the animals that contributed oocytes for ESCs and SCNT-ESCs, contained most of these germ line heteroplasmic mtDNA variants. However, many of these variants in the somatic lineage changed towards homoplasmy and become the main haplotype. We also observed several novel heteroplasmic variants that arose in skin fibroblasts exclusively, albeit they were present at relatively low frequencies (3.4-9.0%). Interestingly, these changes in mtDNA profiles were even more dramatic in muscle and bone marrow mtDNA, most likely reflecting higher metabolic activity in these tissues. Further analysis revealed that many of these somatic homoplasmic variants/mutations were distributed among all mitochondrial genes and resulted in amino acid changes. As expected, mtDNA profiles in iPS cells, in general, reflected heteroplasmy and mutation rates present in parental fibroblasts. We also observed some minor heteroplasmy differences between fibroblast cultures and their iPS cell counterparts, most likely reflecting mtDNA segregation and clonal origin of the latter. *In vitro* differentiation assays demonstrated compromised ability of some iPS cells to generate cardiomyocytes, as compared to their matched SCNT-ESCs. Collectively our results suggest that the germ line mtDNA comprises a wide range of heteroplasmic variants, but at low levels. During development and aging, the frequency of these variants appears to increase in the somatic lineage reaching homoplasmic levels. We also show that iPS cells mirror the variants present in their parental fibroblasts, while SCNT-ESCs regain germ line mtDNA. Since accumulation of mtDNA mutations may significantly compromise the differentiation ability of iPS cells, their potential in modeling human diseases and cell replacement therapies should be carefully considered.

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Poster Board Number: T-3239

ASSESSMENT OF AN IMAGING PROTOCOL FOR THE REAL TIME SELECTION OF HUMAN INDUCED PLURIPOTENT STEM CELL COLONIES USING LIVE CELL MICROSCOPY AND IMAGE RECOGNITION SOFTWARE

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The ability to reprogram somatic cells to an embryonic stem cell-like state has had landmark impact on basic biological research, drug screening, and drug discovery. However, induced pluripotent stem (iPS) cell colony selection remains technically challenging, while characterization of pluripotency is costly and time consuming. Tools that can reliably select fully reprogrammed iPS colonies in real time would be extremely useful to standardize selection, and drive down costs through the selection and expansion of fewer colonies. In addition, it is desirable that such a tool could work using only brightfield imaging as not all reprogramming technologies make use of reporter genes and thus can generate fluorescence images. Previously, we have created a custom image recognition analytic using CL-Quant (Nikon Corporation, Yokohama) for the classification of human iPS colonies in fibroblasts undergoing reprogramming using Klf4, Oct3/4, Sox2 and c-Myc, and achieved a classification accuracy of 99.83% (just 7 errors from 4077 colonies) using only the phase contrast images without fluorescence information. In this study, we have acquired 9 time-lapse, 20 x 20 composite image sequences of the complete 10cm dish at 2x and 4x magnifications. These image sequences show the reprogramming of Spinal Muscular Atrophy (SMA) fibroblast lines imaged at 6 hour intervals for four weeks using a cell culture observation system, BioStation CT (Nikon Corporation, Yokohama). To assess CL-Quant classification performance, iPS-like colonies are selected manually at four weeks post transduction on the basis of their morphological appearance and viral-GFP silencing. We assess 1) the best time point for CL-Quant selection using single time point metrics, 2) the improvement (if any) in selection accuracy from using temporal metrics characterizing the colonies over time from their earliest formation events, and 3) the most useful temporal metrics for colony selection and illustrate their time courses using data and images. Our hypothesis is that incorporating temporal metrics can improve the performance over single point metrics alone in selection of fully reprogrammed iPS colonies. In conclusion, we present an optimal imaging protocol for the real time selection of iPS colonies.

Poster Board Number: T-3240

DERIVATION OF MURINE-ES-CELL LIKE IPS CELLS IN CANINE UNDER THE EOS REPORTER SYSTEM

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Somatic cells can be reprogrammed into induced pluripotent stem cells (iPSC) by ectopic expressions of 4 transcriptional factors (Oct3/4, Sox2, Klf4, c-Myc). It has been difficult to establish iPSC lines in various species other than mice and human. In canine, iPSC lines have been reported and expected to provide for the use of regenelative medicine and of disease-model animals. Canine iPSC lines in the previous reports are similar to human embryonic stem cells (ESC) and have a limited pluripotency. In this experiment, EOS-GFP system, which is controled by Oct3/4 enhancer and early transposone promoter specifically expressed in early mouse embryos, has been used to select iPSC cell lines with more undifferentiated state like murine-ESC. Canine fetal fibroblasts were transfected with Oct3/4, Sox2, Klf4 and c-Myc genes by retrovirus vectors, and were also transfected with EOS-GFP reporter by lentivirus vector. Two weeks after infection, GFP-expressing colonies were obtained, picked up by tripsinaization and then re-seeded onto freshly prepared feeder layers. Resultant canine iPSC-like colonies had round and compact mophology with the expression of EOS-GFP. Canine iPSC expressed pluripotency-related genes and had alkaline phosphatase (AP) activity, and differentiated to all three germ layers *in vitro*. ciPSC easily differenciated by the treatment with JAK inhibitor, which inhibits LIF/STAT signaling. These results suggest that the establised canine iPSC lines have similar characteristics to those of murine ESC regarding morpholorgy, gene expressions, embryo body formation and the dependency on LIF/STAT signaling.

Poster Board Number: T-3241

DNA METHYLATION ANALYSIS OF MOUSE INDUCED PLURIPOTENT STEM CELLS USING BISULPHATE TREATED WHOLE-GENOME SEQUENCING.

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iPS cells have several advantages for stem cell research and regenerative medicine including a potentially limitless supply of patient-matched cells for cell-based therapies without the requirement for human embryonic donor tissue. iPS cells may be used for therapeutics, *in vitro* modelling of human complex diseases, and drug development. Somatic cell reprogramming involves epigenetic remodelling of chromatin architecture including methylation, conferring iPS cells with characteristics similar to embryonic stem (ES) cells. However, it remains unknown whether the re-establishment of an ES-cell-like DNA methylation pattern occurs throughout the genome, and how the methylation pattern changes during the reprogramming process. To address these questions, we utilized a secondary inducible reprogramming system developed in mouse using piggyBac-mediated transposition of the four reprogramming factors[1]. Here we report whole-genome DNA methylation profiles at single-base resolution for mouse ES cells, secondary mouse embryonic fibroblast (MEF), secondary factor independent iPS cells induced from these MEFs, and cells undergoing reprogramming at Day 8, using whole genome bisulphite sequencing. Hiseq 2000 was used for the whole genome sequencing, and Bismark was used as an alignment tool. We defined differentially methylated regions (DMRs) and partially methylated domains(PMDs) in CpG, CHG, and CHH context. iPS cells show significant reprogramming variability, including somatic memory and aberrant reprogramming of DNA methylation. 1. Woltjen K, Michael IP, Mohseni Pet al. piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. Nature. 2009; 458:766-770.

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Poster Board Number: T-3242

PROLIFERATION, MORPHOLOGY AND UNDIFFERENTIATION OF MURINE IPS CELL IN ALGINATE BASE MICROCAPSULE

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Scalable culture systems for propagation/differentiation of induced pluripotent stem cells (iPSCs) are strongly expected for their uses in regenerative medicine or cell-based drug screening. Generally, stirred suspension bioreactors are widely used in mass production, but their direct use for iPSCs culture and differentiation usually results in unignorable cellular damage by shear stress or extensive and inhomogeneous cell aggregation that causes uncontrollable differentiation. To overcome these problems, some researchers reported the hydrogel-based encapsulation. However, these reports are still in a preliminary stage and few reports refer to the biological effect of microcapsule to iPSCs differentiation. In previous ISSCR meeting, we showed three types of micro ($\Phi \approx$ 600 μm) encapsulation system, calcium alginate gel with or without alginate-PLL layers, and with or without EDTA treatment (NAKED group, COATED group, and HOLLOW group respectively)[1], and that iPSCs leaked from the NAKED beads. In this report, we evaluated leakage and undifferentiation of iPSCs in hydro-gel based microcapsule. We encapsulated mouse iPSCs (iPS-MEF-Ng-20D-17; Riken BRC Cell Bank) that have EGFP under the control of Nanog promoter. After 10 days culture, DNA amount measurement and RT-PCR were performed. As a control, same evaluation was performed in direct suspension culture (SUSPENDED group). In the NAKED group, cell leakage was observed and we obtained ununiform aggregates (250-900 μm) outside and small uniform aggregate (100-300 μm) inside. After 10 days, cells expanded 3-fold but 30% of cells were leaked from the NAKED beads. The large ($\Phi \approx$ 1 mm) NAKED beads could only just regulate cell leakage to some extent (10-15% leakage). On the other hand, that leakage was remarkably suppressed by PLL coating (2-3% leakage). It showed that NAKED beads are not suitable for mass culture and investigating biological effect of microcapsule because of the cellular leakage from the capsules, which few reports refer to. We obtained 6 times of cells from COATED beads and 9 times of cells from HOLLOW beads and SUSPENDED. In COATED beads, cells made different size of lens-shape aggregates (300-600 μm). This is because cells were surrounded by hydro-gel and couldn't get together. On the other hand, uniform spherical aggregates (400-550 μm) were observed in HOLLOW beads, because cells could move and get together in the HOLLOW beads. On the other hand, these experiments showed issue of low expansion for mass culture, which is common to microencapsulation. In order to apply microencapsulation for mass culture, it is necessary to modify material or encapsulation process, which promote cell growth. In SUSPENSION system, cells expanded more quickly than encapsulated systems. However, various sizes of aggregates (150-350 μm) and some large cell clumps (550-1000 μm), which lost nanog-GFP fluorescence, were observed. RT-PCR experiment and nanog-GFP fluorescence observation showed that cells kept undifferentiated for 10 days in each type of capsules, but decrease of undifferentiation marker gene expression was observed in SUSPENDED group. Throughout these experiments, we concluded that microencapsulation is better to keep undifferentiation than suspension system but expansion is not enough for mass culture. Now we are investigating early differentiation in microcapsule with a PLL layer.

Poster Board Number: T-3243

CYCLIN D1 GENE EXPRESSION IN MOUSE BMMSCS, ESCS AND IPSCS

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Cyclin D1 is one of important molecule involved in alteration of cell cycle regulators in normal and cancer cells. Expression of Cyclin D1 can be attributed to many factors including increased transcription, translation, and protein stability. Cyclin D1 plays roles as well as a key sensor and integrator of extracellular signals of cells in S to G1 phase that mediate its function through binding the CDKs for modulating local chromatin structure of the genes that are functions in regulation of cell proliferation and differentiation. Although many studies reported that Cyclin D1 is frequently overexpressed in cancers, but its expression and biological functions in stem cells are not clear. Cell reprogramming can convert somatic cells to ESC-like cells that was named "induced pluripotent stem cells (iPSCs)", by using direct transduction of a cocktail composed of only four pluripotent transcription factors: Oct4, Sox2, Klf4 and c-Myc. We speculate that Cyclin D1 may play novel function in cell reprogramming processes and in regulation of cell proliferation and differentiation in stem cells. In our study, we performed Cyclin D1 gene expression in mouse induced pluripotent stem cells (miPSCs), mouse embryonic stem cells (mESCs) and mouse bone marrow-derived mesenchymal stromal cells (mBMMSCs) with comparing mouse embryonic fibroblasts (MEF) using quantitative reveres transcription polymerase chain reaction (qRT-PCR). To compare MEF for Cyclin D1 mRNA level, 54% of expression in mBMMSCs and 28% of expression in mESCs were accounted respectivly. A significant decreased Cyclin D1 mRNA expression was detected in iPSCs. Our results indicated an alteration of Cyclin D1 gene expression in different pluripotency of stem cells. We hypothesize that Cyclin D1 gene expression would be inhibited by novel mechanisms in processes of cell pluripotency and reprogramming.

Poster Board Number: T-3244

PROMOTION OF MOUSE IPSC GENERATION BY HIGHLY EXPRESSED GENES IN BOTH ESCS AND OOCYTES

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Somatic nuclei are reprogrammed by fusion with embryonic stem cells (ESCs) and nuclear transfer into an enucleated oocyte, suggesting that ESCs and oocytes contain a factor capable of reprogramming somatic cells. In recent works, reprogramming of somatic cells to a pluripotent state was achieved by defined factors which are essential to the ESC identity. However, the defined factors-mediated reprogramming was shown to be lower efficiency and slower kinetics than that of the cell fusion- and the nuclear transfer-mediated reprogramming. In addition, comprehensive analyses revealed that induced pluripotent stem cells (iPSCs) generated by four transcription factors (4F; Oct3/4, Sox2, Klf4, and c-Myc)-based method are very similar but not identical to ESCs. These results prompt us to hypothesize that any additional factors are required for efficient and proper iPSC generation. Here we demonstrate that

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mouse iPSC generation is promoted by highly expressed genes in both ESCs and oocytes. Using the BioGPS database, we identified genes highly expressed in mouse ESCs and oocytes, and cloned 39 candidate genes into the pMXs retrovirus vector. Mouse iPSCs were generated from mouse embryonic fibroblasts (MEFs) containing the Nanog-GFP-IRES-Puro^r reporter by retroviral transduction of 4F and an additional gene. We counted the numbers of GFP-positive colonies after 12 days transduction, and found that 6 genes could promote miPSC generation over six fold compared with the control. We next examined whether the gene X, which promotes miPSC generation most efficiently and reproducibly among these six genes, was associated with 4F. Co-immuneprecipitation assay showed that the gene X was associated with Sox2, Klf4, and c-Myc but not with Oct3/4, suggesting that the gene X promotes miPSC generation through Sox2, Klf4, and c-Myc. These findings propose that genes highly expressed in both ESCs and oocytes have iPSC generation-promoting potency and provide new insights into the mechanisms underlying somatic cell reprogramming.

Poster Board Number: T-3245

A COMPARISON BETWEEN IPSCS AND ESCS REVEALS REPROGRAMMING-ASSOCIATED POINT MUTATIONS

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In order to address the issue of whether iPS cells generation is mutagenic, we compared the point mutation profiles between iPSCs and ESCs with genome-wide sequencing. To exclude factors that could influence point mutation analysis, we established ESC clones and genome integration-free iPSC clones under identical culture condition besides the use of feeder for ESCs generation.. Two iPSC clones, 2A-4F-118 and 2A-4F-136, and two ESC lines, B6ES2-2 and B6ES2-9, were used for current study, and their doubling times were indistinguishable and germline transmission was verified for all cell lines. Genome sequences including intergenic regions were determined with pair-end method using HiSeq2000. Consequently, over 90% of entire genome were covered and 55% of it were analyzed at least 10-fold redundancy. Although it must be needed to obtain the corresponding parent and donor cells for identifying single nucleotide variations (SNVs) in iPSCs or ESCs, in human obtaining of them for ESCs is almost impossible. In the present analysis, we employed an inbred strain, C57BL/6J, of which whole genome sequences have been determined, and collected corresponding parent and donor cells in advance. As a result of the genome sequencing of samples and their corresponding parent cells, we could compare 31.9-46.4% of entire genome with high redundancy. 269 and 307 for 2A-4F-118 and 2A-4F-136, and 31 and 13 for B6ES2-2 and B6ES2-9 were thereby identified. All SNVs showed a heterozygous pattern in iPSCs and ESCs but showed a homozygous pattern in the corresponding donor embryo and cells. Next, we conducted Sanger sequencing on 104 SNVs candidates randomly chosen from the pool of candidates for testing our informatics screening and then all of them were verified; all candidates were observed in ESCs or iPSCs in a heterozygous fashion, but not detected in their parent cells. We therefore concluded that SNV candidates predicted by informatics were true SNVs. Our data demonstrate 8.7-23.6 fold higher frequency in iPSCs than ESCs, and suggest the presence of more than 1,000 point mutations in iPSCs genomes. Furthermore, surprisingly, we found unique point mutation profiles in iPSC genomes. Point mutation profiles of iPSCs exhibit transversion-predominant, in contrast, those of ESCs exhibit transition preference. Although in ESCs 30.8-35.5% of point mutations were transversion, in iPSCs those increased by 52.8-57.3%. While transition was also revealed to be increased, transversion rate was increased drastically. In addition, transversion preference was observed only during genome reprogramming, was not observed before and after the reprogramming process. Our observation through the comparison between iPSCs and ESCs clearly demonstrated that iPSCs generation is mutagenic, and the presence of reprogramming-associated point mutations that occur in a transversion-predominant manner. This shed light on the mechanism of iPSCs generation. While this raise concerns for the potential for the clinical use of iPSCs, a mechanistic understanding could resolve the issue.

Poster Board Number: T-3246

INVOLVEMENT OF ADRENERGIC RECEPTORS ON PROLIFERATION AND DIFFERENTIATION OF MOUSE INDUCED PLURIPOTENT STEM CELLS

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[Introduction] Previous studies reported that adrenaline stimulates the proliferation of mouse embryonic stem (ES) cells through adrenergic receptors (AR). Mouse induced pluripotent stem (iPS) cells display properties of self-renewal and pluripotency similar to ES cells, but the effects of AR on the proliferation of iPS cells remains unknown. On the other hand, it was reported that cyclic AMP (cAMP) activation in mesenchymal stem cells may induce neural differentiation. As it is known that activation of β-AR increases cAMP formation, it is hypothesized that stimulation with β-AR on mouse iPS cell may be involved in neural differentiation. In the present study, we examined whether AR stimulation regulates the proliferation or neural differentiation of mouse iPS cells. [Materials and Methods] Mouse iPS cells were cultured under feeder-free conditions in the presence of leukemia inhibitory factor (LIF). Twenty-four hours after re-plating, the cells were treated by l-adrenaline, l-phenylephrine (α1-AR agonist), or prazosin (α1-AR antagonist) for 8~24 h. Proliferation or DNA synthesis of mouse iPS cells was examined by MTT assay or by BrdU incorporation assay. Mouse iPS cell differentiation was initiated by embryoid body (EB) formation under LIF-free condition.. The EBs were stimulated with all trans retinoic acid (ATRA; 1 μM) and/or l-isoproterenol (β-AR agonist; 0.1~3 μM) for 4 days and then transferred to fibronectin-coated dishes. The differentiation potential from mouse iPS cells into neural progenitor cells was evaluated by Nestin expression using immunofluorescence staining or western blot analysis. [Results] The stimulation with l-adrenaline or l-phenylephrine (300 nM) significantly enhanced the DNA synthesis and proliferation of the mouse iPS cells. Pretreatment with prazosin (1 μM) significantly reduced the DNA synthesis and proliferation enhanced by l-adrenaline or l-phenylephrine. Although treatment with l-isoproterenol (3 μM) alone did not affect Nestin expression in the differentiated cells, the treatment significantly enhanced ATRA-induced Nestin expression. The effect of l-isoproterenol was significantly inhibited by pretreatment with atenolol (a specific β1-AR antagonist; 1 μM). [Conclusion] These results suggest that α1-AR stimulation may enhance the proliferation of mouse iPS cells and β1-AR stimulation may enhance the neural differentiation of the cells.

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Poster Board Number: T-3247

A COMPARISON BETWEEN IMMUNOGENICITY OF IPSCS AND OF ESCS

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Recently, an important study that showed an immunogenicity of induced pluripotent stem cells (iPSCs) was reported. Using an inbred mouse strain, C57BL/6, induced T-cell-dependent immune response to teratomas was demonstrated in syngenic recipients. Here we established many lines of embryonic stem cells (ESCs) in addition to integration-free iPSCs from C57BL/6 for obtaining a conclusion on this issue. Fully reprogrammed state was verified by germline transmission test for the most lines. First, we performed teratoma formation test. Slightly efficient formation was observed in iPSCs, but basically little difference in incidence was observed between iPSCs and ESCs. Furthermore, we could not detect meaningful T -cell-infiltrarion not only in the teratomas derived from iPSCs but also from ESCs. In addition, we examined the expression of Homad1 and Zg16, but their expressions in the teratomas derived from iPSCs were lower than those in ESCs. Thus, unexpectedly, even using a large number of ESCs and iPSCs, we could not detect any differences between these two types of pluripotent stem cells. Second, we assessed the immunogenicity of the differentiated cells derived from iPSCs and ESCs, dermal tissue and bone marrow. Donor tissues were prepared from 2N chimera and/or 4N chimera mice; to completely exclude the recipient derived cells from donor tissues, we used 100% chimera only that were generated by aggregation with GFP-mice embryos. The tissues that were confirmed to be GFP-negative, were transplanted. Consequently, even in the cases focusing on differentiated cells, we also observed little difference not only in incidence but also in T-cell response; almost all transplantations were succeeded and very few T-cells were observed within the transplanted tissues. Additionally, transplantation of bone marrow cells into recipient mice without X-ray irradiation, was also succeeded and hematopoietic reconstitution was achieved two months later. Thus, in the present study we could not observe immunogenicity of iPSCs and iPSCs-derived tissues. There is little difference in immunogenicity between iPSCs and ESCs .

Poster Board Number: T-3248

NEURONAL DIFFERENTIATION OF MOUSE INDUCED PLURIPOTENT STEM CELLS USING CONDITION MEDIUM OF DORSAL ROOT GANGLION AND ROCK INHIBITOR

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Mouse induced pluripotent stem (iPS) cells were directly generated from mouse fibroblasts by introducing four genes, *Oct3/4*, *Sox2*, *c-Myc*, and *Klf4*. The iPS cells exhibit the morphology and growth properties of embryonic stem (ES) cells and ES cell marker genes, and may have a pluripotent ability to differentiate into various cell lineages *in vitro* including neurons. The *in vitro* differentiation of iPS cells provides new perspectives for studying the cellular and molecular mechanisms of early development and the generation of donor cells for transplantation therapies. Because we have already reported the differentiation of mouse ES cells into neurons using conditioned medium of dorsal root ganglia (DRG-CM), we investigated the differentiation of iPS cells into neurons using DRG-CM and ROCK inhibitor. Recently, it was reported that ROCK inhibitor (Y-27632), a specific inhibitor for Rho-dependent protein kinase (ROCK), permits the survival of dissociated human ES cells by efficiently blocking the dissociation-induced cell death (apoptosis). Mouse iPS cells (APS0001; RIKEN Cell Bank, Saitama, Japan) were grown on a mitotically inactivated mouse embryonic fibroblast feeder layer in the presence of leukemia inhibitory factor (LIF). The iPS cells appeared to be maintained in an undifferentiated state until passage 17-19. To form iPS cell colonies, iPS cells were transferred to nonadhesive plastic dishes. The number of iPS cell colonies (approximately 200 μm in diameter) increased until 6 d of cultivation. To achieve the directed differentiation of iPS cells into neurons, iPS cell colonies were cultivated in DMEM/F-12K medium containing DRG-CM. The iPS cell colonies effectively differentiated into neurons when they were cultivated for 6-12 days with the culture medium containing 5-10% DRG-CM. Gene expression analysis using RT-PCR appeared that nestin (a marker for neural stem cell) gradually increased and TH (a marker for dopaminergic neuron) increased at day 9. On the other hand, we investigated the growth of dissociated iPS cells by the addition of Y-27632. When iPS cells were plated 500 cells/well with a growth medium containing 10 μM of Y-27632, the iPS cells proliferated very well. Then, we investigated the differentiation of dissociated iPS cells into neurons by the addition of Y-27632. Dissociated iPS cells were plated 5000 cells/well in a gelatin-coated 96-well culture plate with a differentiation medium containing 10 μM of Y-27632. After 12 days of cultivation, many iPS cells differentiated into neurons compared to control. In addition, the iPS cells efficiently differentiated into neurons by the addition of both 10 μM of Y-27632 and 10 ng/ml of nerve growth factor (NGF). These results show that iPS cells could be effectively induced to differentiate into neurons using DRG-CM in a similar manner as ES cells and the addition of Y-27632 to the culture medium is useful to the growth and differentiation of iPS cells.

Poster Board Number: T-3249

MOUSE IPS CELL TRANSPLANTATION WITH WHISKER STIMULATION INCREASES ENDOGENOUS MIGRATION IN A NEONATAL STROKE MODEL

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Stroke is a leading cause of death and disability world wide. More specifically, neonatal stroke affects 26 of 100,000 live births each year, yet there are limited treatments available to repair stroke-damaged tissue. Induced pluripotent stem (iPS) cell transplantation is currently being explored as a possible therapeutic. In previous work, transplantation of embryonic stem (ES) cells has been shown to increase the recruitment of progenitors to the infarct. Similarly, in our previous work using the whisker barrel cortex stroke model, we demonstrated that whisker stimulation also increases progenitor migration to the infarct area suggesting that increasing thalamocortical afferent signals to the damaged barrel cortex may serve as a guiding signal to migrating progenitors for regeneration. Therefore, we hypothesize that iPS cells and whisker stimulation together promote migration and neurogenesis in a neonatal rat stroke model. Whisker barrel cortex mini-stroke was induced in P7 neonatal rat pups through a temporary occlusion of the common carotid artery and permanent occlusion of the middle cerebral artery. Seven days after stroke, 400,000 iPS cell-derived neural

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progenitor cells were transplanted into the stroke penumbra. The rats received daily BrdU injections to label proliferating cells and whisker stimulations 3 times a day for 5 minutes each. Rats were sacrificed 21 days after stroke and examined for BrdU co-labeled with NeuN (neuronal marker) or Collagen IV (vessel marker) in the infarct penumbra. Pluripotent iPS cells were stained for trophic factor expression *in vitro* and terminally differentiated iPS cell-derived neurons were patch-clamped to record sodium and potassium currents. Rats transplanted with iPS cell-derived progenitors exhibited greater numbers of BrdU-, NeuN/ BrdU-, and collagen IV/ BrdU-labeled cells in the penumbra compared to rats with stroke only. Rats that received both whisker stimulation and transplantation had the greatest number of BrdU-labeled, NeuN/BrdU and collagen IV/BrdU cells in the ischemic penumbra. Immunocytochemistry of iPS cells *in vitro* showed expression of several trophic factors involved in neurogenesis, cell survival, and cell migration including SDF-1, FGF, EPO, and GDNF. Mature iPS cell-derived neurons were patch-clamped and exhibited neuronal functionality with inward sodium and outward potassium currents *in vitro*. iPS cell-derived progenitor transplantation and whisker stimulation may promote neurogenesis and angiogenesis after an ischemic barrel cortex infarct in neonatal rats. The increased NeuN/ BrdU and collagen IV/ BrdU cells suggests increased neurovascular remodeling with whisker stimulation and iPS cell-derived neural progenitor transplantation. Transplantation of iPS cell-derived neural progenitors may contribute additional trophic support that increases endogenous migration to the infarct, endogenous neurogenesis, and transplant cell survival. The combination of whisker stimulation and transplantation therapy shows great potential as a synergistic therapeutic strategy for neonatal ischemic stroke.

Poster Board Number: T-3250

A HIERARCHY OF REPROGRAMMING POTENCY IN MOUSE LIVER

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Ectopic expression of certain transcription factors can reprogram somatic cells to a pluripotent state. It has been shown that hematopoietic stem cells can be reprogrammed with a higher efficiency than differentiated blood cells. Similar findings have not been demonstrated in other primary organ systems. Moreover, molecular characteristics in the cellular hierarchy of tissues that influence reprogramming capacities need to be delineated. Here, we analyzed the influence of the differentiation stage of freshly isolated, murine liver cells on the reprogramming efficiency. Liver progenitor cell (LPC)-enriched cell fractions from fetal and adult liver showed a significantly increased reprogramming efficiency after transduction of 3 or 4 reprogramming factors. Transfection efficiency corrected reprogramming rates of fetal LPCs were 275 fold higher compared to un-sorted fetal liver cells when 3 reprogramming factors were transfected. LPC-derived iPS cells showed all hallmark features of pluripotency. The increased reprogramming efficiency of LPCs compared to differentiated liver cells occurred independent of proliferation rates but associated with the endogenous expression of reprogramming factors (Klf4 and c-Myc) and two members of the BAF-complex (Baf155 and Brg1) mediating epigenetic changes during reprogramming. Knockdown of BAF-complex members abolished the increased reprogramming efficiency of LPCs vs. non-LPCs. The current study provides the first functional evidence that LPCs carry intrinsic, cell proliferation-independent characteristics allowing highly efficient reprogramming compared to differentiated liver cells.

Poster Board Number: T-3251

RNA-SEQ ANALYSIS OF MOUSE INDUCED PLURIPOTENT STEM CELLS REVEALS A NEGATIVE ROLE OF CYCLIN D1 IN REGULATING THE GROUND STATE PLURIPOTENCY AND CELL CYCLE ADAPTION

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Pluripotent embryonic stem cells (ESCs) exhibit a unique cell cycle structure characterized by a shorter G1 phase and higher proportion of cells located in S phase. Recent works demonstrated introduction of Oct4, Sox2, cMyc and Klf4 into somatic cells would enforce their returning to pluripotent state. How fidelity of pluripotent cell cycle and self-renewal control can be re-established during reprogramming process remain to be determined. To address the issue, we initially separated early reprogramming cells into three subpopulations based on SSEA1 expression and Oct4 promoter activity. We found only SSEA1+/Oct4-GFP+ cells can give rise to fully reprogrammed induced pluripotent stem cells (iPSCs) and exhibited although SSEA1+/Oct4-GFP- or SSEA1-/Oct4-GFP-cells can proliferate quickly and expressed a number of pluripotent characteristics. Cell cycle analysis revealed SSEA1+/Oct4-GFP+ cells were closely resemble ESCs. RNA-Seq analysis was perform to determine expression profile of cells of somatic cells (TTF fibroblasts), SSEA1-/GFP-, SSEA1+GFP-, SSEA1+GFP+, SSEA1+GFP+ (late passage) and TT2 ESCs. We revealed a number of cell cycle regulators including cyclins D, B, A and Cdk inhibitors were altered during the preprogramming. We further indentified that cyclin D1 & D2 was up-regulated in SSEA1-/GFP-, SSEA1+GFP- but down-regulated in SSEA1+GFP+. Although Cyclin D1 was highly induced by Klf4 and Oct4 and constitutive expressed through the cell reprogramming, it was not required for cell proliferation. Knockdown of Cyclin D1 with shCcnd1 between reprogramming day 0- day 6 significantly reduced formation of alkaline phosphatase-positive and SSEA1-positive colonies confirmed that Cyclin D1 was required for initiation stage. In contrast, inducibly overexpressing Cyclin D1 during reprogramming further revealed Cyclin D1 overexpression at reprogramming day 0- day 6 was only able to enhance formation of SSEA1-positive colonies, but not Oct4-GFP-positive colonies. In contrast, knockdown of Cyclin D1 or addition of CDK4/6 inhibitor, PD0332991, at reprogramming day 8- day 14 could enhance formation of Oct4-GFP-positive colonies indicating that Cyclin D1 play a dual role in pluripotent reprogramming. Importantly, we confirmed both iPSCs and ESCs expressed lower level of Cyclin D1. Overexpression of cyclin D1-T286A mutant in both iPSCs and ESCs suppressed Klf4 and impaired LIF/Stat3 signaling which resulted in loss of identities of the pluripotent ground state. In conclusion, our findings suggested down-regulation of cyclin D1 in the late reprogramming stage is critical for reprogramming cells to return to pluripotent ground state and to re-establish pluripotent cell cycle.

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ESTABLISHMENT OF IPS CELLS FROM AMYOTROPHIC LATERAL SCLEROSIS MODEL MICE AND MOTOR NEURONAL DIFFERENTIATION

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Subject: To unravel pathogenesis of amyotrophic lateral sclerosis (ALS), methods using induced pluripotent stem cells (iPS cells) are promising. We establish iPS cells from ALS model mice, mutant superoxide dismutase1 (SOD1) transgenic mice, induce neural differentiation and unravel pathogenesis of ALS. Methods: We introduced known four factors (Oct3/4, Sox2, Klf4, c-Myc) into mouse embryonic fibroblasts obtained from crossbreeding of SOD1G93A mice with Nanog-GFP-IRES-Puror mice using retroviral vectors and cultured them on SNL feeder cells. We picked ES cell-like colonies, cultured them and confirmed establishment of iPS cells using ES cell markers. Then we induced directed differentiation using retinoic acid and Smoothened agonist and confirmed motor neuronal identity by immunocytochemistry. Results: We established iPS cells from mutant SOD1 mice and control mice and confirmed motor neuronal differentiation. Conclusions: These results indicate that iPS cells from ALS model mice possess properties of ES cells with mutant SOD1 and may contribute to establishment of ALS model(s) *in vitro*.

Poster Board Number: T-3253

COMPARATIVE ANALYSIS OF DOPAMINERGIC NEURON DIFFERENTIATION EFFICACY FROM MOUSE EMBRYONIC STEM CELLS AND PROTEIN-BASED IPS CELLS

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During the progression of Parkinson's disease, stem cells can act as therapeutic agents to recover, or regenerate injured nervous system. In this study, two types of stem cells; mouse embryonic stem cells (mESCs) and protein-derived iPS cells (P-iPSCs), generated by non-viral methods, were differentiated into midbrain dopaminergic (mDA) neurons and compared its efficiency. In the undifferentiated stage, P-iPSCs expressed pluripotency markers without any difference to that of mES cells elucidating the fact that protein-based reprogramming was successful and stable as authentic ES cells. All two types of cells reached terminal matured mDA neurons while P-iPSCs showed more mDA neuron positive markers in protein levels as well as in mRNA levels when compared with mES cells. To investigate the mechanism of significantly advanced induction of mDA neurons in P-iPSCs, we analyzed histone modifications by genome-wide ChIP sequencing analysis and their corresponding microarray results. We found Wnt signaling was up-regulated while SFRP1, known as its counter-actor was suppressed more in P-iPSCs than mESCs. Moreover, dramatic change of expression level of epigenetic regulator, Sirt1 may be associated with differentiation efficiency. In 6-OHDA-induced Parkinson's rat model, both types of transplanted neural precursor cells showed rescued motor activity and migration in damaged striatum. Our results demonstrate that P-iPSCs can be an ultimate source for patient-specific cell therapy and efficient to replace damaged neurons *in vivo* as well.

Poster Board Number: T-3254

DISRUPTED STEM CELL HOMEOSTASIS IN PREMATURELY AGING MICE WITH MTDNA MUTAGENESIS

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A proof-reading deficient mitochondrial DNA polymerase gamma in the transgenic mtDNA mutator mice results in accumulation of point-mutations in the mtDNA, defects in the respiratory chain complex subunits and a mild respiratory chain deficiency in aged mice. The mtDNA mutator mice develop several premature aging symptoms, such as anemia, reduced subcutaneous fat, kyphosis, alopecia, osteoporosis, sarcopenia and reduced life span. The mild respiratory chain phenotype seen in post-mitotic tissues does not explain the aging related symptoms seen in proliferating tissues. We have shown a disrupted somatic stem and progenitor cell homeostasis in mutator neural stem (NSC) and hematopoietic progenitor (HPC) cell compartments. This stem cell dysfunction is present already during embryonic life, long before any symptoms arise, or before any evidence of respiratory chain dysfunction. Further, this stem cell dysfunction can be ameliorated by treatment with antioxidant (N-acetyl-cysteine; NAC), suggesting that an imbalance in the redox regulation or a subtle increase in ROS (reactive oxygen species) production underlies the stem cell phenotype. Based on our results on somatic stem cells, we suggest that the mutator mice have two separate dysfunctions. 1) The mild respiratory chain dysfunction that manifests in post-mitotic tissues of aged mice, and 2) an early imbalance in the cellular ROS/redox regulation that causes somatic stem cell dysfunction. To provide further evidence for a "stemness" defect in mutators we set out to generate induced pluripotent stem (iPS) cells from mutators. While we were successful in reprogramming mutator fibroblasts, the efficiency of reprogramming was significantly reduced when compared to wild-type cells. Further, mutator fibroblasts treated with NAC showed a significant increase in colony formation when compared to untreated cells. The established mutator iPS cell lines had ES-like characteristics, but showed severe growth defect as well as reduced ability to self-renew. While the growth defect was not affected by the antioxidant treatment, the treated mutator iPS lines showed self-renewal ability comparable to wild-type iPS lines. These data indicate that both somatic and pluripotent stem cells, are especially sensitive to mitochondrial dysfunction and that the mechanism involves ROS/redox homeostasis.

Poster Board Number: T-3255

CO-TRANSPLANTATION OF MOUSE IPS DERIVED CARDIOMYOCYTES AND MESENCHYMAL STEM CELLS FOR THE REGENERATION OF INFARCTED MYOCARDIUM

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Introduction: Cardiomyocytes generated from induced pluripotent stem cells (iPS-CM) hold great promise as a sustainable and autologous source of cells with potential for the regeneration of infarcted myocardium. The goal of this study was to analyze the efficacy of a cell transplantation strategy using iPS-CM in combination with

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mesenchymal stem cells (MSC). Methods: A murine iPS cell line was genetically engineered to express antibiotic resistance and EGFP under the control of cardiac α-myosin-heavy-chain promoter and, additionally, firefly luciferase driven by a constitutive ubiquitin C promoter. iPS-CM were generated by puromycin selection in a mass culture differentiation system and their purity and quality was assessed by flow cytometry, immunocytochemistry and qPCR. Murine MSC were isolated from bone marrow and cell identity was confirmed by *in vitro* differentiation and flow cytometry. A myocardial infarction was induced in syngeneic mice by cryoinjury followed by intra-myocardial transplantation of $5x10^5$ iPS-CM, MSC or a combination of both. The retention of cells over the course of four weeks was assessed by *in vivo* bioluminescent imaging of iPS-CM and detection of paramagnetically labeled MSC using a clinical 3T magnetic resonance imaging (MRI) scanner. MRI was also used for the assessment of cardiac pump function (left ventricular ejection fraction, LV-EF) at weekly intervals. Immunohistochemical analyses were performed to locate the transplanted cells on a microscopic level. Results: Puromycin-selected iPS-CM were more than 95% pure, devoid of contaminating pluripotent stem cells and did not cause teratoma formation in immunodeficient animals. iPS-CM expressed α-actinin and cardiac troponin T and exhibited the sarcomeric organization typical of immature cardiomyocytes. MSC expressed CD44 (64.1±4.1%) and Sca-1 (98.1±0.9%) and showed *in vitro* adipogenic, chodrogenic and osteogenic differentiation potential. After transplantation into cryo-infarcted hearts LV-EF increased significantly for iPS-CM transplanted and iPS-CM/MSC co-transplanted animals as compared to MSC transplanted and sham operated animals (LV-EF, four weeks after transplantation - sham: 44.2±2.6%; iPS-CM: 51.8±3.3%; MSC: 47.6±1.9%; iPS-CM+MSC: 55.7±2.3%; sham vs. iPS-CM, sham vs. iPS-CM+MSC, MSC vs. iPS-CM+MSC, all $p<0.001$; n=8). Paramagnetically labeled MSC could be detected within the site of transplantation in the ventricular wall for four weeks. The bioluminescent signal from iPS-CM decreased within the first week below the level of detection. However, immunohistochemical analyses of hearts four weeks after transplantation revealed the presence of patches of EGFP-, α-actinin- and connexin 43-positive iPS-CM, indicating structural maturation and functional integration. Conclusion: Intramyocardial co-transplantation of iPS-CM and MSC into infarcted hearts resulted in improved recovery of heart function, as compared to transplantation of single cell populations, demonstrating the potential of combined transplantations of iPS cell-derived cardiac cells and adult stem cells for regenerative therapies. However, large fractions of transplanted iPS-CM were lost within the first week after injection, either due to their poor survival or mechanical extrusion from the site of injection. Therefore, additional optimization is required for achieving improved engraftment of transplanted cells and better therapeutic efficacy.

Poster Board Number: T-3256

EXPERIMENTAL TRANSPLANTATION OF HUMAN IPS CELLS DERIVED NEURAL CELLS IN A MOUSE MODEL OF HEMIPLEGIA.

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[Introduction] Stroke is the leading cause of adult disability due to the limited repair capacity of neural cells. Neural cell transplantation is a promising strategy to overcome this limitation. We have found that monkey embryonic stem (ES) cell derived neural cell transplantation were effective to restore the motor functions of stroke model mice. In this study, we tried to generate neurons from human inducible pluripotent stem (hiPS) cells and to transplant the cells to hemiplegic model mice. [Method] 1. Culture of hiPS cells: A hiPS cell line was obtained from RIKEN Cell Bank (cell name: 253G1, cell number: HPS0002) and was maintained according to RIKEN cell preparation manual. 2. Cell differentiation: We developed embryonic bodies (EBs) from undifferentiated hiPS cells by 4-day floating culture. EBs were transferred to fibronectin-coated dishes and cultured for 24 hours. We added retinoic acid, sonic hedgehock and noggin in the dishes twice and cultured for 72 hours. RT-PCR analysis and immunocytochemistry were performed to evaluate their differentiation. 3. Stroke mouse model and cell transplantation: Cryogenic injury was made in motor cortex by the direct compression of a chilled metal probe to a frontal bone at a point where the bone was dug with a drill in advance. One week later, the burr hole mark was made near the probe compression point and hiPS cell derived neural cells were injected into periventricular area under the injured motor cortex layer. We used the rotarod test and the beam walking test to evaluate the motor functions before and after transplantation. Three weeks later from the transplantation, we conducted pathological analysis and immunohistochemistry. For statistical analysis, MANOVA was used and $p<0.05$ was considered as significant. [Results] 1. Cell differentiation: The cells derived from hiPS cells expressed neural markers in RT-PCR and confocal immunofluorescence analysis as well. The former showed that the cells expressed genes of beta III tubulin, nestin, neurofilament and Islet1. The latter disclosed that the cells were 30-50% neural cell adhesion molecule (NCAM) positive, 90% nestin positive and 90% beta III tublin positive. 2. Transplantation: All mice showed severe hemiplegia after cryogenic injury. The hiPS cell derived neural cells survived and migrated to the injured motor cortex from the transplanted area. Motor functions were significantly improved in the beam walking test throughout the observation period. [Conclusions] We successfully generated hiPS cell derived neural cells which retained their function after transplantation *in vivo*. We found that the clinical application of hiPS cell derived neural cells looked promising to restore the motor functions of patients with hemiplegia.

Poster Board Number: T-3257

4D-NUCLEOFECTOR™ SYSTEM: CELLULAR REPROGRAMMING WITH AN EYE TOWARDS THE CLINIC

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Pluripotent stem cells hold great promise for cell replacement therapies. While human embryonic stem cells (hESCs) are limited to allogeneic therapies, human induced pluripotent stem cells (hiPSCs) can be applied to both allogeneic and autologous therapies; the latter having the advantage of utilizing a patient's own cells. Recent advances in the field have shown that various adult cell types can be reprogrammed to a pluripotent state by introduction of defined transcription factors. This process of cellular reprogramming, however, has been robust only when the transcription factors are delivered by viruses thereby diminishing applicability of hiPSCs to cell therapy. Lonza's Pluripotent Stem Cell Innovation Center is focused on developing tools and technologies that apply to both research and clinical applications. For the latter, the generation of hiPSCs under cGMP conditions is a necessity. As an important first step towards cGMP-grade hiPSCs, the example shown below combines Lonza's 4D-Nucleofector™ system with a "zero-footprint" reprogramming technology. Here we demonstrate efficient generation of hiPSCs from blood cells using the 4D Nucleofector™

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System. Lonza hiPSCs share characteristics with human embryonic stem cells (hESCs), including the hESC-associated markers POU5F1, NANOG, TRA-1-81 and TRA-1-60. In addition, Lonza hiPSCs show alkaline phosphatase activity, a normal karyotype, and the ability to efficiently differentiate into cells of all three germ layers. Notably, Lonza iPSCs show no trace of exogenous DNA integration, confirming that cells were reprogrammed with a "zero footprint" technology. These results contribute to Lonza's goal of generating iPSCs under cGMP conditions for cell therapy applications.

Poster Board Number: T-3258

SUPPRESSED NEUTROPHIL DEVELOPMENT IN HEMATOPOIESIS OF INDUCED PLURIPOTENT STEM CELLS DERIVED FROM A SEVERE CONGENITAL NEUTROPENIA PATIENT WITH ELA2 MUTATION

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Severe congenital neutropenia (SCN) is a rare disorder characterized by severe neutropenia present at birth, an arrest of neutrophilic differentiation at the promyelocyte or myelocyte stage, and a propensity to develop acute myeloid leukemia and myelodysplasia. Mutations of the ELA2 gene encoding neutrophil elastase have been identified in the majority cases of SCN, but the mechanisms which disrupt neutrophil development in SCN with ELA2 mutation have been unclear. To understand the mechanisms, we established three human induced pluripotent stem (iPS) cell clones (SPN0101, SPN0102 and SPN0103) from bone marrow stromal cells of a patient having heterozygous mutation in ELA2 gene at exon 5, 707 region by transfection with retrovirus vector which expressed human OCT3/4, SOX2, KLF4, and c-MYC (SCN-iPS cells). The silencing of exogenous genes and the capability to differentiate into three germ layers by teratoma formation were confirmed in the three SCN-iPS clones. We also identified the ELA2 gene mutation in SCN-iPS cells same to that in the patient. We then examined the hematopoietic differentiation of SCN-iPS and control iPS cells which were generated from healthy donors by the same method to SCN-iPS cells, using coculture system with a murine stromal cell line (AGM-3 cells). The cocultured cells were harvested at day 12, and CD34+ cells were separated. Hematopoietic colony assay was performed using these CD34+ cells. Although number and size of erythroid and mixed-lineage colonies derived from SCN-iPS cells were almost similar to control, those of myeloid colonies derived from SCN-iPS cells were significantly less and smaller than control. In particular, we could detect few number of G colonies from SCN-iPS cells. Since SCN patients need granulocyte colony-stimulating factor (G-CSF) treatment to increase peripheral neutrophils, we conducted the hematopoietic colony assay with G-CSF alone to examine the sensitivity of granulopoiesis derived from SCN-iPS cells and control iPS cells to G-CSF. Meloid colony formation reached a plateau at 1 to 10 ng / mL of G-CSF in control iPS cells, while the number and size of myeloid colonies gradually increased at up to 1000 ng / mL in SCN-iPS cells, but did not attain the control level. In suspension culture with myeloid differentiation-oriented cytokines including 10 ng / mL of G-CSF, CD34+ cells from control iPS cell increased 23.3-fold for 2 weeks, and mature neutrophils predominantly occupied in the cultured cells. By contrast, CD34+ cells from SCN-iPS cells gradually decreased, and few neutrophils, but mainly monocytic cells were contained in the culture. We finally carried out microarray analysis using CD34+ cells stimulated by myeloid differentiation-oriented cytokines for 2 days to identify the genes which led to impaired granulopoiesis in SCN-iPS cells. As a result, LEF-1, C/EBP alpha, Cyclin D1 and BCL2 were downregulated in the cultured cells from SCN-iPS cells compared with those from control iPS cells. These results demonstrated that the development of neutrophils was selectively impaired in the hematopoiesis derived from SCN-iPS cells, and that the stimulation of higher concentration of G-CSF compensated the impaired development of neutrophils to some extent, indicating SCN-iPS cells can provide a useful tool to understand pathogenesis of SCN with ELA2 mutation.

Poster Board Number: T-3259

PATIENT DERIVED IPSC TO STUDY ALPORT'S DISEASE

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Alport's Syndrome is an inherited disease caused by mutations in COL4A3, COL4A4 and COL4A5 genes which code for type IV collagen α3, α4 and α5 chain genes, respectively. Approximately 85% of Alport's cases are X-linked dominant, caused by mutations in the COL4A5 gene. The disease is associated with glomerulonephritis, endstage kidney disease, and hearing loss due to loss of basement membrane function. There is no known cure and treatment options are symptomatic only. With the aim of generating an *in vitro* model of the disease we generated provirus free iPSC from fibroblasts obtained from a skin biopsy of a patient with Alport's Syndrome. iPSC were generated using a CRE excisable six factor polycistronic lentiviral reprogramming cassette that we developed and colonies were picked between day 14 and 18 of reprogramming. The provirus was excised using CRE-RNA and excision was confirmed by absence of a PCR product for the lentiviral backbone and the SOX-2/KLF4 junction in the reprogramming plasmid. Both iPSC and provirus iPSC were fully characterized for stem cell pluripotency including endogenous gene expression, immunofluorescent imaging of colonies, EB formation, teratoma formation of all three germ layers and routine karyotype analysis. The genotype of the patient was unknown, so after extensive sequencing of the 6,455 Kbp COL4A5 gene we discovered a novel frame shift mutation, 2598delG. This mutation leads to truncation of the COL4A5 protein from 1690 amino acids to 816 amino acids. The Alport's iPSC at early passage in particular have a distinct phenotype, the iPSC initially attach to matrigel and then ball up with a tendency to form EBs. Passage with Accutase and ROCK inhibitor is able to prevent this from occurring. Furthermore, EB's are poorly defined. Alport's Syndrome is associated with an increase in matrix metalloproteinase production, particularly MMP9. Using zymography we assessed the activity of gelatinases, including MMP9 a 92 kDa type IV collagenases or gelatinase B, and found a significant increase in MMP9 activity in Alport's patient derived iPSC when compared to wild type iPSC ($P<0.05$). As wild-type bone marrow derived cells have been shown to significantly improve renal function in a COL4A3-/- mouse model

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of Alport's Syndrome we differentiated the iPSC into mesenchymal stem cells. These cells were fully characterized using a panel of markers by Flow Cytometry and for their capability to differentiate to adipocytes, osteoblasts and chondrocytes. These disease-linked iPSC-derived mesenchymal stem cells enable further study of the mechanisms pertaining to Alport's disease and to assess their potential for therapeutic approaches for COL4A5 deficiency. In conclusion we have generated the first iPSC from a patient with Alport's Syndrome. Correction of this novel disease mutation via homologous recombination advances the development of cell based therapies and create a valuable congenic positive control for *in vitro* models of this disease.

Poster Board Number: T-3260

MODELING OF ALPERS SYNDROME CAUSED BY POLG MUTATION BY INDUCED PLURIPOTENT STEM CELLS

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The fibroblasts from one patient with Alpers syndrome caused by POLG (mitochondrial DNA polymerase gamma subunit) mutation were isolated, and the induced pluripotent stem cells were derived from the patient specific fibroblasts (AHS-iPS). Then, with a 21-day, 5-staged *in vitro* development protocol, the AHS-iPS was induced towards hepatocyte-like cells displaying various hepatic functions. As controls, hES and normal iPS were also induced towards hepatocyte-like cells. Based on these works, the defective phenotypes of mitochondria are investigated in the hepatocyte-like cells. Further, by treating the hepatocyte-like cells with valproic acid, the underlying mechanism of AHS would explain the onset of lethal acute liver failure in AHS patients.

Poster Board Number: T-3261

MODELING VASCULITIS SYNDROME USING MICROSCOPIC POLYANGIITIS MPA SPECIFIC IPSCS

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Microscopic polyangiitis (MPA) is an autoimmune disease characterized by pauci-immune, necrotizing, small-vessel vasculitis without clinical or pathological evidence of necrotizing granulomatous inflammation. The disorder presents with rapidly progressive glomerulonephritis as the most frequent manifestation, while pulmonary hemorrhage is among the most life-threatening complications. Although previous reports describe that the activation of neutrophils and vascular endothelial cells is involved in the development of vascular inflammation in animal models, the mechanisms of vasculitis remain largely unknown in human. Here we report the derivation of induced pluripotent stem cells (iPSCs) from skin fibroblasts of three patients with MPA by retroviral transduction of four transcription factors: OCT3/4, SOX2, KLF4 and c-MYC, or three factors: OCT3/4, SOX2 and KLF4. MPA-iPSCs are similar to human embryonic stem cells (ESCs) in their morphology, cell behavior and the expression of surface antigens and marker genes for the undifferentiated state. In addition, the disease-specific iPSCs show multipotent differentiation ability into three embryonic germ layers both *in vitro* and *in vivo.* Notably, we have confirmed that MPA-iPSCs can be induced to differentiate into vascular endothelia and neutrophils *in vitro,* cell types associated with the pathogenesis of MPA. Furthermore, the iPSC-derived endothelia and neutrophils showed some aspects of immunological functions involving the production of cytokines/chemokines. These results suggest that MPA-iPSCs can be used to create a novel *in vitro* disease model for vasculitis syndrome, supplying resources to study disease mechanisms, screen new drug compounds and develop novel therapeutic strategies.

Poster Board Number: T-3262

GENERATION OF IPS CELLS FROM PATIENT FIBROBLASTS CARRYING MITOCHONDRIAL RESPIRATORY CHAIN COMPLEX DEFECT

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Mitochondrion is a cellular power plant producing energy necessary for cellular metabolism. Impaired mitochondrial respiratory activity causes numerous disorders from metabolic defects to severe and fatal symptom. Although various mutations on mitochondrial respiratory chain complex have been reported, metabolic mechanisms of m.3398T>C mutation on MTND1 gene have been poorly understood. In this study, fibroblasts of a mitochondrial respiratory chain complex disease patient carrying m.3398T>C mutation were reprogrammed to the pluripotent state by ectopic expression of four transcription factors (OCT4, SOX2, c-MYC, and KLF4). These disease-specific iPS cells with normal karyotypes expressed pluripotency markers at transcription and protein levels. Also, it was confirmed by DNA methylation analysis that promoter regions of endogenous pluripotency marker genes were epigenetically reprogrammed in the iPS cells. Like human ES cells, the disease-specific iPS cells had immature mitochondrial cristae. These iPS cells can be used as a cellular model for studying metabolic mechanisms of the mitochondria-related disease in human.

Poster Board Number: T-3263

HUMAN INDUCED PLURIPOTENT STEM CELLS: MODELING PARKINSON'S DISEASE

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Parkinson's disease (PD), the second-most common neurodegenerative disease, is characterized by the degeneration of dopaminergic neurons in the substantia nigra pars compacta in the midbrain. Patients present with a loss of TH^+ neurons, as well as aggregation of the protein α-synuclein in the surviving dopamine neurons. Because the majority of PD cases are sporadic, it is often defined as an idiopathic syndrome. However, about five percent of PD cases are considered familial and are characterized by the presence of known mutations in genes related to the development of PD. Studies into finding underlying factors for the development of PD are hindered by the lack of a relevant human model. The derivation of iPSCs from PD patients provides us with a starting point for developing a human *in vitro* model of the disease. The study of this model may provide clues into the molecular mechanisms that underlie the disease. We obtained fibroblasts from patients with a multi-generational family history of PD, as well as from patients with sporadic PD. Fibroblasts from a non-PD patient were also obtained, for use

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as a control. These patient fibroblasts were transformed using a lentivirus carrying Yamanaka's transcription factors. We were able to successfully derive and characterize the resultant induced pluripotent stem cells (iPSCs). Under neural differentiation conditions, the iPSC lines were able to reach the neural precursor stage, expressing relevant early neural precursor markers, as well as beginning to form neural rosettes. The availability of these iPSC lines will give us the opportunity to evaluate, side by side, if there are any differences in neutralization capacity, ability to form dopaminergic neurons, or susceptibility to exogenous culture chemicals. These results may provide insights on the mechanisms underlying the development of PD.

Poster Board Number: T-3264

INDUCED PLURIPOTENT STEM CELLS DERIVED FROM IDIOPATHIC PARKINSON'S DISEASE PATIENTS DIFFERENTIATE INTO MIDBRAIN DOPAMINERGIC NEURONS

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Induced pluripotent stem cells (iPSCs) are promising source for cell replacement therapies. However, several problems remain to be solved before they can be used in clinical settings. Such problems include the use of animal-derived materials such as feeder cells, immune rejection by the hosts, and re-activation of virally transfected genes. As for immune rejection, autologous transplantation of iPSCs derived from somatic cells of patients can be a solution. Recent studies showed that iPSCs derived from patients with sporadic Parkinson's disease (PD) could differentiate into dopaminergic (DA) neurons. Upon transplantation into PD model rats, they successfully reduced amphetamine- and apomorphine-induced rotational movements, indicating their contribution to functional recovery. In this study, we show that iPSCs derived from patients with idiopathic PD can differentiate into dopaminergic neurons using our feeder-free floating culture method. First we generated iPSCs from the dermal fibroblasts of these patients by reprogramming with episomal vectors. Polymerase chain reaction (PCR) confirmed that resulting iPSCs had no genomic integration of those vectors. On the day 0 of neural induction, pluripotent iPSCs were seeded onto 96-well plates in GMEM supplemented with 8% KSR. With the addition of Nodal and BMP inhibitors from day 0 to day 12, most of the cells in the floating culture were positive for nestin, an early neural marker, at day 12. From day 12 onwards, the medium was replaced with Neurobasal medium supplemented with B-27, and DA neurons were induced by the addition of Purmorphamine, fibroblast growth factor 8 (FGF8), brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), dibutyryl cyclic AMP, and Ascorbic acid. Immunohistochemistry and quantitative PCR indicated midbrain DA neuronal identity of the generated neurons. In conclusion, we differentiated iPSCs derived from PD patients into midbrain DA neurons with our feeder-free differentiation method. iPSCs generated with episomal vector method and our floating culture method with minimal animal-derived materials would be more suitable for clinical settings than reported methods.

Poster Board Number: T-3265

PATIENT-SPECIFIC STEM CELL-DERIVED MODELS FOR ALZHEIMER`S DISEASE - A DANISH CONSORTIUM

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The groundbreaking finding that somatic cells can be reprogrammed into iPS cells has opened up for novel ways of identifying patient specific medicine. The technology is a step towards development of personalized medicine and is expected to increase the low proportion (<10%) of drug candidates, entering clinical studies for central nervous system (CNS) disorders. A focused Danish Consortium has recently initiated its development of *in vitro* models for well-characterized Alzheimer disease (AD) patients based on iPS technology. The Consortium involves leading pharmaceutical competences within neurodegeneration as well as leading clinical and scientific competences within neural development, pluripotent stem cells and disease modeling. The overall goal for the Consortium is to establish a pipeline all the way from the AD patient to the *in vitro*-derived mature neuron and its use for targeted drug screening compatible with a pharmaceutical screening approach. The Consortium will be focusing on AD patients with identified mutations like e.g. PSEN1/2 and MAPT. Proper selection of optimal subpopulations of patient fibroblasts for reprogramming, based on epigenetic and marker characteristics, will be an important first step in the process. Controlled expansion (optimal O_2 tensions and growth factor addition) of subpopulations of either patient derived fibroblasts- or blood (CD34+) cells for integration-free reprogramming (miRNA/episomal based) has already been established (see Holst *et al* poster). Transfection of normal human dermal fibroblasts (NHDF; Lonza) was compared between different liposome-based methods including Lipofectamine 2000 (invitrogen), X-tremeGene (Roche), and MagnetoFection (OZ Biosciences), and a Neon electroporation device (Invitrogen). Results were evaluated by flow cytometry using constitutively expressed RFP or GFP. The Neon electroporation method yielded superior transfection efficiencies of >60% with lower overall cell death (50%) when using a single pulse (1600 V for 20 ms). This method is currently being tested with episomal vectors (Yu et al., 2009), with and without P53 siRNA (Okita et al., 2011), Minicircles (Jia et al., 2010), or a Minicircle containing the miR-302/607 cluster (Anokye-Danso et al., 2011). The ratio of iPS-like cells is analyzed by flow cytometry every week over a 4-week period using the pluripotency markers SSEA-3/4 and Tra-1-60. The number of iPSC-like colonies will eventually be visualized by live staining with Tra-1-81and picked. Another major task of the Consortium is to establish an efficient and reproducible differentiation process to cholinergic neurons in a xenofree and defined environment. The first differentiation trials have demonstrated that human iPSCs within cultured in a xenofree environment can be differentiated towards the neuroepithelial lineage. In our trials (combining SB431542 and Noggin) a strong upregulation of the early neural marker, PAX6, has been observed at both mRNA (qPCR, >100 fold) and protein level. Exchange of Noggin with a specific analogue is being explored as the final xenofree process for the establishment of neuroepithelial cell populations intended for further cholinergic directed differentiation of AD derived iPSCs. We believe that the involvement of strong scientific, clinical and pharmaceutical partners in the Consortium will pave the way for a

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process, generating AD specific *in vitro* models as well as discoveries of new AD disease mechanisms.

Poster Board Number: T-3266

INVESTIGATION OF THE PATHOGENESIS OF PARKINSON'S DISEASE BY PD PATIENTS DERIVED-INDUCED PLURIPOTENT STEM CELLS

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Parkinson's disease (PD) is one of the common neurodegenerative diseases. It is preferentially happened to elderly people, and leads to the movement disorder. The hallmarks of PD are progressive loss of midbrain dopaminergic neurons and the appearance of Lewy bodies in the affected neurons. At present, the etiology of PD is not well understood. Some familial forms of PD are caused by mutations on the PD-related genes, Parkin, PINK1, LRRK2, and SNCA. However, most of the cases of PD are idiopathic and may reflect the interactions of environmental and genetic factors. The hurdle of studying PD pathogenesis is the inaccessibility of brain tissues from PD patients. Recent advances in cellular reprogramming now allow an alternative approach for investigating PD. PD patients' skin fibroblasts can be converted to the pluripotent state, and further differentiated to the specific disease cell type, dopaminergic neurons. Such PD affected neurons in culture represent a promising departure point for exploring the pathogenic factors of PD. Here we have generated disease specific human induced pluripotent stem cells (hiPSCs) from 3 individual idiopathic PD patients' fibroblasts and one healthy person fibroblast. We applied a single lentiviral vector (hSTEMCCA-loxP) which encodes OCT4, KLF4, SOX2, and c-MYC, and subsequently discarded the transgenic plasmid using cre-loxP recombination. The genomic constitutions of PD-hiPSCs were found identical to their parental fibroblasts by DNA fingerprinting. The characteristics of PD-hiPSCs resemble human embryonic stem cells (hESCs) with the expression of pluripotency marker genes and the capability of differentiating into 3 germ layers. Moreover, some of the PD-hiPSCs passed the teratoma tests, 3 different lineage tissues could be observed *in vivo*. Neural precursors were derived from PD-hiPSCs, and then applied for transcriptome analysis. Our results revealed some differentially expressed genes involved in calcium homeostasis and neural development. In the future work these genes will be examined in PD derived neural precursors and differentiated dopaminergic neurons. Meanwhile, PD affected dopaminergic neurons will be analyzed in morphology and function compared to normal dopaminergic neurons in order to discover novel molecular players involved in Parkinson's disease.

Poster Board Number: T-3267

DEVELOPMENT OF A NEW METHOD: THE CLONING FOR THE HEREDITARY UNKNOWN DISEASE GENE USING PATIENT-DERIVED IPS CELLS.

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Linkage analysis is a well-established and powerful method for mapping disease genes of Mendelian inheritance. Although linkage analysis is a powerful tool, it depends on the number of pedigree information. So analysis with large number of pedigrees points out few candidate genes. While one or two families analysis produces many candidate genes. Recently, it is possible to scan of the personal whole genome using the next generation sequencer. Although this new technology finds some DNA base changes rapidly and efficiently, these results cannot tell us whether the DNA base change is the disease mutation or very rare variant in small numbers of family analysis. To address this problem, we generate the patient-derived iPS cell and try to identify disease gene from one family only.

Poster Board Number: T-3268

UTILIZING PATIENT-SPECIFIC INDUCED PLURIPOTENT STEM CELLS TO MODEL ALS

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Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease that affects motor neurons in the brain and spinal cord. Generation of treatments for ALS and the understanding of mechanisms that cause the disease have been hampered by an inability to culture motor neurons isolated from patients and by the complex genetic nature of most cases. Patient-specific induced pluripotent stem (iPS) cells, generated by epigenetic reprogramming of somatic cells, provide an invaluable tool for *in vitro* disease modeling, and could be used to address these problems. We have previously shown that iPS cells are similarly variable in their genomic qualities to ES cells as well as in their ability to differentiate into physiologically active motor neurons. Here we addressed whether iPS cells can be used to probe ALS in a cell culture system. We specifically focused on familial ALS cases that carry a mutation in the gene SOD1, which is responsible for around 25% of all genetic ALS. We show that motor neurons derived from these lines exhibit a variety of phenotypic differences to ones generated from healthy individuals. In particular, ALS-motor neurons decrease in numbers over long-term culture, with the largest motor neurons being selectively lost. Although ALS-derived motor neurons do not form large SOD1 aggregates, a typical pathological phenotype in post mortem patients, they exhibit signs of ER stress and mitochondrial impairment as well as altered electrophysiological properties. These results, for the first time provide an insight to the functional defects that physiological levels of mutant SOD1 may lead to in patient motor neurons and broadly demonstrate that iPS cell technology can be used to probe an adult-onset neurological disease such as ALS.

Poster Board Number: T-3269

DERIVATION OF AUTISM SPECTRUM DISORDER-SPECIFIC INDUCED PLURIPOTENT STEM CELLS FROM PERIPHERAL BLOOD MONONUCLEAR CELLS

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Autism spectrum disorders (ASDs) comprise an array of complex neurodevelopmental disorders. One of the major constraints in ASD research is the lack applicable human disease models. Patient-specific induced pluripotent stem cells (iPSCs) hold great potential for deciphering the molecular pathophysiology of human genetic disease by giving rise to genetically relevant disease-affected cell

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types that can be studied *in vitro*. iPSCs are most commonly derived from fibroblasts, which are obtained through skin biopsies. Among certain populations of individuals, including children with ASDs, the necessity for skin punch biopsies to extract fibroblasts make them an unsuitable source of cells for reprogramming. To circumvent this limitation, we have derived iPSC lines from peripheral blood mononuclear cells (PMBCs) isolated from the whole blood of autistic children. Furthermore, the autism iPSCs could differentiate into GABAergic and dopaminergic neurons, which are neural subtypes frequently affected in ASDs.

Poster Board Number: T-3270

PATIENT-SPECIFIC INDUCED PLURIPOTENT STEM CELLS FROM CARDIAC PROGENITORS RECAPITULATE THE MODELS FOR CARDIAC CHAMBER DISORDERS

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Background- In contrast to human, zebrafish can efficiently regenerate the lost cardiac muscles shortly after injury. Although the studies have indicated the genetic fingerprint essential for self-repair in lower vertebrate with single ventricle, yet, there is little model for investigating the candidate genes potentially contributed to cardiac regeneration in human heart. Single ventricle (SV) physiology is a rare but fatal congenital heart defect in human. Here, we propose that the creation of disease-specific induced pluripotent stem (iPS) cells from patients with congenital heart malformation allows investigation on disease phenotype and may provide an opportunity to uncover the genetic evidence for myocyte renewal in human. Methods- Disease-specific iPS cells were generated from 15 patients include 10 patients with SV physiology and 5 bi-ventricle (BV) control hearts. Myocardial tissue specimens were minced to isolate cardiac progenitor cells (CPCs) and the cells were infected with a combination of retroviruses encoding the human transcription factors Oct3/4, Sox2, Klf4, and c-myc. Alkaline phosphatase staining and immunofluorescent analysis were performed to verify the authenticity of generated iPS clones. RNA was isolated from the cells for quantitative PCR and whole-genome expression analysis. Bisulfite sequencing was assessed by using the primers amplify the Oct3/4 and Nanog promoter regions. Results- In culture, CPCs from SV hearts showed an enhanced proliferative potential compared with those from BV patients. Although iPS colonies from most individuals can be readily identified by embryonic stem (ES) cell-like morphology, ES cell-like colonies formed from SV-derived CPCs can be expanded more efficiently than those from BV patients (70% vs. 20% of subjects analyzed). Bisulfite sequencing analysis showed that Oct3/4 and Nanog promoter regions were demethylated in both types of iPS cells generated. Likewise, iPS cells from CPCs could formed teratoma when injected the cells into immunocompromised mice. Whole genome analysis revealed that Nkx2-5, Tbx5, and Mef2c, those are core transcriptional networks involved in mammalian heart development, were comparable between the CPCs isolated from SV and BV patients. We also found that inductive signals specific for second heart field development, including Isl1, Hand1, Wnt3a, BMP typeI receptor, and Bop1 were significantly downregulated in SV-derived CPCs; however, Notch1 and Foxh1 expressions increased remarkably compared with BV-derived CPCs. Surprisingly, GATA4 which is responsible for zebrafish heart regeneration after injury was decreased in SV-derived CPCs. Both types of CPCs demonstrated similar calcium oscillation before lineage induction. Upon cardiac differentiation, transcriptional factors include alpha-MHC, Nkx2-5, Tbx5, Hand2, and Isl1 were significantly upregulated in BV-derived iPS cells, whereas these genes remained unchanged but GATA4 expression was enhanced in SV-derived iPS cells. Conclusions- Our results suggest that factor-based reprogramming of patient-derived cardiac progenitors can efficiently generate disease-specific iPS cells. CPCs and iPS cells from single ventricle hearts have distinct characteristics during cardiac differentiation. Verification of genes prerequisite for zebrafish heart regeneration by using iPS cells may enable disease investigation and also facilitate the development of new cardiac regeneration therapy in human.

Poster Board Number: T-3271

DISEASE CHARACTERIZATION USING LONG QT SYNDROME SPECIFIC INDUCED PLURIPOTENT STEM CELLS

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[Background] Although previous reports have indicated that long QT syndrome-specific iPS cells (LQTS-iPSCs)-derived cardiomyocytes recapitulated disease phenotypes, those patients were previously diagnosed with mutated channel profiles. In reality, most patients have no such specific information. To address whether iPSCs could be used for personalized disease characterization, we generated iPSCs from a sporadic LQTS patient with unknown disease cause. [Methods and Results] (1) We generated iPSCs from control (n = 2) and a patient with LQTS, and differentiated into cardiomyocytes through embryoid body (EB) formation. (2) Electrophysiological analysis of the LQTS-iPSCs-derived EBs using multi electrode array system revealed that the duration of the field potential (FPD) was markedly prolonged compared with the control (388.9 ± 44.3 msec vs 202.3 ± 16.3 msec, $P<0.01$). (3) We tested several drugs affecting QT prolongation to clarify the electrophysiological properties. The IKr blocker E4031 significantly prolonged FPD (% change 1.21 ± 0.02, $P<0.01$) and induced frequent severe arrhythmia, not only early-after depolarization (n = 8/16 vs n = 1/16) but also torsades de pointes-like arrhythmia, only in LQTS (n = 4/16 vs n = 0/16). The IKs blocker, chromanol 293B did not prolong FPD in LQTS but it significantly prolonged FPD in the control (% change 1.09 ± 0.04, NS vs 1.44 ± 0.07, $P<0.01$), suggesting the involvement of IKs disturbance in the patient. Isoproterenol induced ventricular tachycardia-like arrhythmia only in LQTS, which was blocked by propranolol. These data strongly suggested a functional impairment in the patient's IKs channel; genotype analysis for KCNQ1 gene revealed a novel heterozygous mutation, 1893delC. (4) Patch clamp analysis and immunostaining confirmed a dominant-negative role for 1893delC in IKs channel through a trafficking deficiency. [Conclusions] LQTS-iPSCs-derived cardiomyocytes recapitulated the disease phenotypes, and they can be utilized for identification of the disease cause and genotype. This study demonstrated that iPSCs could be useful to characterize the disease, drug responses, diagnosis and genotyping in patients with sporadic LQTS, which in turn may facilitate medical therapies in the clinical settings.

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Poster Board Number: T-3272

GENERATION OF INDUCED PLURIPOTENT STEM CELLS FROM PRIMARY HEMATOLOGICAL MALIGNANCIES.

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Induced pluripotent stem cells (iPSCs) can be generated by the expression of defined transcription factors not only from normal tissue, but also from malignant cells. Bone marrow cells from a chronic myelogenous leukemia (CML) patient were reprogrammed by introducing the transcription factors Oct3/4, Sox2, KLF4, and c-myc. Two CML derived iPSCs (CML-iPSCs) were generated. Although CML-iPSCs expressed the bcr-abl, they were resistant to imatinib. Comprehensive analysis of DNA methylation revealed the very similar methylation pattern between normal and CML-iPSCs. Then we differentiated them into hematopoietic progenitors. They showed the hematopoietic marker CD45 and immature marker CD34, and recovered the sensitivity to imatinib, which recapitulated the feature of initial CML disease. The phosphorylation state of ERK1/2, AKT, and STAT5 were evaluated after imatinib treatment in CML-iPSCs. The phosphorylation of ERK1/2 and AKT were unchanged after treatment, whereas the phosphorylation of STAT5 was decreased in CML-iPSCs although it was not activated in normal iPSCs. The phosphorylation of CrkL, which is the direct target of the bcr-abl, was decreased in both CML-iPSCs and CML-iPSCs derived hematopoietic cells although it was not activated in the normal iPSCs. These results showed that the signaling for iPSCs maintenance compensates for the inhibition of bcr-abl in CML-iPSCs and that bcr-abl dependence was lost in CML-iPSCs. Thus, this system can act as a new platform for the elucidation of the disease mechanism and drug screening.

Poster Board Number: T-3273

SPONTANEOUS NEURONAL DIFFERENTIATION IN TUBEROUS SCLEROSIS COMPLEX DERIVED PLURIPOTENT STEM CELLS

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Tuberous Sclerosis Complex (TSC) is a multiorgan disease caused by mutation or deletion of either the *TSC1* (encodes hamartin) or *TSC2* (encodes tuberin) genes. Hamartin and tuberin bind to one another and deficiency of either protein is sufficient to cause TSC. Although multiple downstream signaling pathways appear to be controlled by the *TSC1/2* genes, inhibition of the mTOR kinase appears to be central. Inhibition of the mTOR complex 1 (mTORC1) in fact has become an established and potent therapy for at least a subset of the clinical manifestations seen in TSC. The pathological hallmark of TSC is hamartomas (collections of disorganized non-malignant cells) that appear to underlie the severe disease manifestations that are seen in most patients. Neurological features are particularly severe with many patients suffering from epilepsy, mental retardation and autism. Pathological examination of brain hamartomas ("tubers") reveal large neurons and glial cells that are highly dysmorphic. The prevailing model of TSC pathogenesis posits a germline mutation in *TSC1/2* genes followed by a "second hit" mutation/deletion in the other *TSC1* or *TSC2* allele. Such loss of heterozygosity is then required for the formation of hamartomas in brain ("tubers"), kidney, heart, lung and skin. While experimental support for this model has been verified with non-brain hamartomas, loss of heterozygosity in cortical tubers has been extremely difficult or not possible to demonstrate. To study the role of the *TSC1/TSC2* genes during human development and to begin to unravel complex genotype/phenotype relationships and the role of mTORC1 inhibition, we have generated induced pluripotent stem cells (iPSC) from multiple patients with TSC using both viral and plasmid-based reprogramming methods. Biochemical and genetic analyses indicate increased mTORC1 signaling in TSC patient derived iPSC. TSC patient derived iPSC exhibited rapid growth and spontaneously generated immature neurons even under stem cell maintenance conditions. Compared to control iPSC, spontaneously generated neurons were much more frequently seen and had elongated, multipolar neurites. Treatment with the mTORC1 inhibitor rapamycin reversed these phenotypes. In addition, TSC patient derived iPSC had altered media requirements for growth factors in a cell-autonomous manner. Finally, loss of heterozygosity was not found in TSC patient derived iPSC lines. These last findings are highly significant as it suggests that abnormal differentiation of human neural progenitor cells and neurons may be due to haploinsufficiency of the *TSC1* or *TSC2* genes. If validated, these findings would possibly change therapeutic approaches to the treatment of TSC and related disorders.

Poster Board Number: T-3274

IN VITRO MODELING OF NEUTROPHIL DEVELOPMENT AND FUNCTION USING IPSCS: CLINICAL VALUES IN UNDERSTANDING THE PATHOPHYSIOLOGY AND APPLICATIONS IN THE TREATMENT OF CHRONIC GRANULOMATOUS DISEASE.

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Introduction: Neutrophil (NEU) differentiation is a dynamic process that can be recaptured in vitro using induced pluripotent stem cells (iPSCs). This process is susceptible to manipulation by cytokines, allowing for the study of these cells at specific stages in their development. Most importantly from a clinical perspective, by generating patient autologous iPSCs, it is possible to recapitulate a specific disease phenotype. Chronic granulomatous disease (CGD) is a congenital NEU disorder characterized by the impaired generation of reactive oxygen species (ROS). The transplantation of gene modified CD34+ cells offers the best hope of a permanent cure but carries the inherent risk of genotoxicity. In this study, we aim to highlight the clinical value of iPSCs as a disease model in elucidating the underlying pathophysiology and in assessing the effective recovery of cell functions following gene modification.
Methods: Peripheral blood (PB) CD34+ cells were isolated from two CGD patients with gp91phox or p47phox deficiency and from a healthy donor. Cells were reprogrammed using a Sendai virus vector expressing Oct4/Sox2/Klf4/c-Myc. Self-inactivating lentiviral or alpharetroviral vectors were used to insert either gp91 or p47 cDNA into iPSCs. Neutrophil differentiation was induced using VEGF and

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G-CSF. WG, MPO and ALP staining was done to assess cell morphology. The immunophenotypic profiles of differentiating cells were assessed by staining for macrophage and neutrophil specific antigens. ROS production was analyzed by the DHR flow cytometry assay following NEU stimulation with PMA. Neutrophil extracellular traps (NETs) were visualized by staining with SYTO 13 and anti-MPO antibody. Neutrophils isolated from healthy donors served as the control. Results: Mature PB-NEUs and control iPSC-NEUs displayed the classic multi-lobed appearance of the nucleus. ROS were generated at comparable levels and both populations were able to form NETs. However, CGD iPSC-derived NEUs (gp91phox and p47) appeared to show impaired development. This was suggested by the fact that at day 7 of the differentiation culture, only a proportion of the population stained positive for ALP, which is a protein found in secretory vesicles. At the same time point, nearly all control iPSC-NEUs stained positive for ALP. CGD iPSC-derived NEUs were ROS negative at all time points and displayed impaired formation of NETs. The loss of these functions however was recovered in NEUs differentiated from gene modified CGD iPSCs. Conclusion: This is the first report to show that in vitro differentiated NEUs have the capacity to form NETs. Only mature NEUs with a complete repertoire of cellular components and normal ROS generating capacity possess this property. Along with other matching characteristics to PB NEUs, these results may be taken as validation of the accuracy with which this model may be utilized to mimic NEU physiology and development *in vivo*. Indeed, in this instance it has been utilized to uncover a previously unreported impairment in CGD NEU development. Further study is required to determine the implications of this. In addition, we have shown that vector mediated gene transfer can recover the characteristic loss of function associated with CGD NEUs. It is possible that this disease model may also be used to study insertion site profiling for example. This will allow therapeutic vectors to be evaluated for clinical safety thus minimizing the potential risk of genotoxicity and possible harm to patients.

Poster Board Number: T-3275

HIGH THROUGHPUT SCREENING OF NEUTRAL LIPID STORAGE DISEASE, MYOPATHY SUBTYPE IPS CELLS IDENTIFIES MODULATORS OF INTRACELLULAR LIPID METABOLISM THAT REDUCE DISEASE PHENOTYPE *IN VIVO*.

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Induced pluripotent stem cells (iPSCs) hold great promise as in vitro disease models for pathway discovery and therapeutic screening. Here, we generate a murine iPSC model of neutral lipid storage disease, myopathy subtype (NLSD-M) (also know as Triglyceride Cardiomyovasculopathy (TGCV)), a condition characterized by a marked elevation of lipid accumulation in cardiac and skeletal muscle due to the loss of functional adipose triglyceride lipase (ATGL) that converts triglyceride to diacylglycerol for the production of ATP by fatty acid β-oxidation. Using a high throughput screening (HTS) platform for differentiated iPSCs we identified several small molecule modulators of cellular metabolism that normalize the intracellular lipid level in differentiated NLSD-M murine iPSCs. Further characterization of these compounds revealed their induction of glycolysis and suppression of oxidative respiration. To validate the lipid modulatory effects of these compounds *in vivo*, we treated NLSD-M mice with three of the top-hits identified in our HTS screen (mefloquine, perhexiline, and vinpocetine). Over an 18-week study period, we found all three of these drugs reduced triglyceride accumulation in the heart, improved cardiac function, and prolonged survival by 4 weeks (i.e. ~33% increase in lifespan) when compared with untreated NLSD-M mice. Preliminary gene expression analyses show a normalization of cardiac metabolic enzyme levels in drug treated hearts. Our results represent the first successful HTS screening of disease iPSCs for the discovery of novel therapy. Given the increased prevalence of NLSD-M/TGCV in Japanese population, our drugs may benefit these patients when examined in a clinical setting.

Poster Board Number: T-3276

GENERATION OF INDUCED PLURIPOTENT STEM CELLS FOR THE STUDY OF AUTISM SPECTRUM DISORDERS

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Autism spectrum disorder, normally exhibits the onset of symptoms before 3 years of age, and is characterized by severe impairment in reciprocal socialization, impairment in communication skills, and repetitive or restrictive behaviors. It is a heterogeneous condition of multiple etiologies; no precise clinical assessment tools currently allow precise definition between the multiple variants, nor are there biological markers to distinguish these variants. A rise in the number of children identified with autism spectrum disorders, from five to 72 cases per 10,000 children in the USA and Europe, and the absence of definitive forms of therapy have resulted in increased public concern. Improved strategies for early identification of specific phenotypic characteristics and biological markers (e.g., electrophysiological changes) hopefully might improve the effectiveness of treatment. The invasive nature of collecting primary neuronal tissue from patients might be circumvented through the use of iPSC and their subsequent neuronal differentiation. With the successful reprogramming of human fibroblasts into ES cell-like state (aka induced pluripotent stem cells, iPSC) by Yamanaka et al in 2007, this methodology has subsequently been successfully employed to derive cultured neural cells from patients with ALS, Parkinson disease, and other disorders. These breakthroughs make it possible for us to generate a cell culture model of autism spectrum disorder by application of iPSC reprogramming of human fibroblasts and subsequent neural differentiation. In this study, fibroblast cultures from patients (subject with autism), and non-affected controls have been established; subsequently these cells are reprogrammed into an ES cell-like state (aka induced pluripotent stem cells, iPSC). The reprogrammed cell colonies are cloned, propagated, and induced to differentiate *in vitro* into neuronal cultures. Based on our underlying assumption that synaptic transmission is aberrant in autism, these patient-specific neuronal cultures will be utilized for neuronal network analysis by using the photoconductive-stimulation system described in Gutierrez et al. Briefly, spontaneous or pulse-stimulated activity of networks is measured by optical techniques, and the structural basis of these patterns will analyzed by fractal dimension analysis. By use of these approaches we have the capacity to characterize the arrangement and complexity of their axonal architecture. This approach has been

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employed to demonstrate differences in hippocampal cultures of a rat model carrying the neurolignin mutation R471C-NL3 which has been identified in a subgroup of patients with autism spectrum disorders. This study represents the pilot attempt to evaluate membrane excitation and signal transduction in neural cells derived from patients with autism.

Poster Board Number: T-3277

NEURAL DIFFERENTIATION OF MULTIPLE SCLEROSIS IPS CELL LINES

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The recent introduction of technologies capable of reprogramming human somatic cells into induced pluripotent stem (iPS) cells offers a unique opportunity to study many aspects of neurodegenerative diseases *in vitro* that could ultimately lead to novel drug development and testing. Here, we report for the first time that human dermal fibroblasts from a patient with relapsing-remitting Multiple Sclerosis (MS) were reprogrammed to pluripotency by retroviral transduction using defined factors (OCT4, SOX2, KLF4, and c-MYC). The MSiPS cell lines resembled human embryonic stem (hES) cell-like colonies in morphology and gene expression and exhibited silencing of the retroviral transgenes after four passages. MSiPS cells formed embryoid bodies that expressed markers of all three germ layers by immunostaining and Reverse Transcriptase (RT)-PCR. The injection of undifferentiated iPS cell colonies into immunodeficient mice formed teratomas, thereby demonstrating pluripotency. The MSiPS cells were successfully differentiated into mature astrocytes, oligodendrocytes and neurons with normal karyotypes. Although MSiPS-derived neurons displayed some differences in their electrophysiological characteristics as compared to the control cell line, they exhibit properties of functional neurons, with robust resting membrane potentials, large fast tetrodotoxin-sensitive action potentials and voltage-gated sodium currents. This study provides for the first time proof of concept that disease cell lines derived from skin cells obtained from an MS patient can be generated and successfully differentiated into mature neural lineages. This represents an important step in a novel approach for the study of MS pathophysiology, for disease modeling and potential drug discovery.

Poster Board Number: T-3278

MODELING TRISOMY-21 SYNDROME WITH IPS CELLS FROM AMNIOTIC FLUID CELLS

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Trisomy 21(T21) Syndrome is a common chromosomal abnormality caused by an extra copy of chromosome 21 that contributes to the cognitive impairments associated with Down Syndrome (DS). The human gene for amyloid precursor protein (APP) is located on chromosome 21. APP is a ubiquitously expressed transmembrane protein whose cleavage product, the β-amyloid (Aβ) protein, is deposited as amyloid plaques in the aged brain, and in the neurodegenerative conditions of Alzheimer disease's (AD) and DS. Recent study demonstrated that in DS patient's, neurons overexpress miR-155 and miR-802 lead to low levels of the target protein, methyl-CpG-binding protein (MeCP2), which is important for the maturation of neuron cells. Generation of diseased induced pluripotent stem (iPS) cells has unique values in developing human genetic models *in vitro*. This technology holds the promise of increased understanding of complex disease. Here we have generated human iPS cell lines from second trimester amniotic fluid (AF) cells with T21 by lentiviral delivery of Yamanaka factors. We report that T21 AF-iPS cells were characterized for expression of pluripotent markers such as alkaline phosphatase activity, Oct4, Sox2, Nanog, TRA-1-60, TRA-1-81, SSEA-3, SSEA-4 and the abilities of differentiation into three germ layers by forming embryoid bodies *in vitro* and teratomas *in vivo*. The T21 AF-iPS still maintained the abnormal and unique chromosomal karyotypes, three pairs of chromosome 21. We found the expressed protein level of APP in neuron progenitor cells (NPCs) derived from T21 AF-iPS cells to be significantly increased compare with NPCs from normal AF-iPS cells. We also demonstrate that the expression of miR-155 and miR-802 in T21 AF-iPS-NPCs were highly expressed in presence low expression of MeCP2. Our findings demonstrate that T21 AF-iPS cells serve as a good source to further elucidate neurogenesis of DS and onset of Alzheimer's disease.

Poster Board Number: T-3279

MOLECULAR ANALYSIS OF FMR1 REACTIVATION IN FRAGILE-X INDUCED PLURIPOTENT STEM CELLS AND THEIR DERIVED NEURONS

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Patient specific induced pluripotent stem (iPS) cells, generated from somatic cells of disease affected individuals, hold a tremendous potential for studying disease mechanisms and for drug screening approaches using cell types not previously available. Fragile-X (FX) syndrome belongs to the autistic spectrum disorders, and is the most common cause of inherited mental retardation. It is nearly always caused by silencing of the FMR1 gene due to abnormal CGG repeat expansions in the 5'-UTR of the gene. Abnormal CGG repeat expansion of over 200 repeats leads to transcriptional silencing and CpG methylation of the gene 5'-UTR and the gene promoter. Recently, we have generated 11 FX-iPS cell lines from 3 different FX patients. In FX-iPS cells, the gene is transcriptionally silent both in the pluripotent and differentiated state. The absence of FMR1 expression in FX-iPS cells is accompanied by DNA methylation and histone modifications indicative of heterochromatin at the gene promoter. Here, we wished to evaluate the reactivation of FMR1 in FX-iPS cells and their differentiated neuronal derivatives by epigenetic modulating drugs. We find that 5-azacytidine (5-azaC) was able to robustly reactivate gene expression both in FX-iPS and FX-neuronal cells. This reactivation is coupled with extensive DNA demethylation of the gene promoter as well as increasing levels of histone H3 acetylation and histone H3K4 methylation, but not decreasing levels of H3K9 methylation. Finally, we show that FMR1 gene expression is maintained even when 5-azaC treatment is withdrawn. This is the first time that reactivation of FMR1 is shown in stem cells or neuronal cells of FX patients by a pharmacological treatment. These findings may eventually pave the way to affect the progression of the disease in FX patients.

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Poster Board Number: T-3280

MODELING FOR AUTOSOMAL RECESSIVE POLYCYSTIC KIDNEY DISEASE USING PATIENT-SPECIFIC IPSCS.

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Autosomal recessive polycystic kidney disease (ARPKD) is one of the most common pediatric renal cystic diseases, with an estimated incidence of 1 in 20,000 live births. The disorder is characterized by non-obstructive fusiform dilatations of the renal collecting ducts resulting in enlarged spongiform kidneys and ductal plate malformation of the liver leading to congenital hepatic fibrosis. Most patients with ARPKD present perinatally with oligohydramnios caused by decreased fetal urine output and related hypoplastic lungs, but others present later in life when the clinical symptomatology is dominated by either renal failure or hepatic dysfunction, or both. The pathogenesis of congenital hepatic fibrosis as well as renal cyst formation remains largely unknown, and no therapeutic strategies have been established. Here we have generated induced pluripotent stem cells (iPSCs) from skin fibroblast samples of a 10-year-old female patient with ARPKD by transducing five transcription factors, OCT4, SOX2, KLF4, L-MYC and LIN28 with non-integrating episomal vectors. ARPKD-iPSCs are similar to human embryonic stem cells (ESCs) in their morphology, cell behavior, and the expression of surface antigens and marker genes for the undifferentiated state. In addition, the disease-specific iPSCs show multipotent differentiation ability into three embryonic germ layers both *in vitro* and *in vivo.* Notably, we have confirmed that ARPKD-iPSCs can be induced to differentiate into bile duct epithelia and renal collecting duct cells *in vitro,* cell types affected in ARPKD. Using these differentiation systems and microarray analyses, we are identifying the molecules involved in the onset or progression of liver fibrosis and cystic kidneys by comparing the expression profiles of the hepatic and renal cells differentiated from ARPKD-iPSCs with those from normal Japanese iPSCs. These results suggest that disease modeling using patient-specific iPSCs can be used for studying the molecular mechanisms in the pathogenesis of ARPKD.

Poster Board Number: T-3281

REAL TIME SCORING OF HUMAN INDUCED PLURIPOTENT STEM CELL DIFFERENTIATION POTENTIAL USING LIVE CELL MICROSCOPY AND IMAGE RECOGNITION SOFTWARE

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The ability to reprogram somatic cells to an embryonic stem cell-like state has had landmark impact on basic biological research, drug screening, and drug discovery. Ultimately, achieving the promise of induced pluripotent stem (iPS) cell research requires the controlled differentiation of cells to ecific cell types. However, it is well known that there can be large differences in target cell type yields for iPS lines even from the same patient. Previously, we have created an image recognition tool that can be used to classify iPS-like colonies in microscopy image sequences with a high degree of accuracy, and in a related poster here we more thoroughly assess an imaging protocol for the automated selection of fully reprogrammed iPS colonies using live cell microscopy and image recognition. Another question we are interested in is whether these technologies can be used to score iPS colonies, both fully and partially reprogrammed (piPS, viral-GFP positive colonies with iPS like morphology), for having a differentiation bias towards specific cell types in real time. In this study, we have acquired 8 time-lapse, 20 x 20 composite image sequences of the complete 10cm dish at 2x and 4x magnifications showing the reprogramming of colonies from fibroblast lines of Spinal Muscular Atrophy patients imaged at 6 hour intervals for four weeks using a cell culture observation system, BioStation CT (Nikon). 40 iPS-like and piPS colonies were then picked expanded and differentiated towards cardiomyocyte lineages in embryoid bodies and their differentiation propensities assessed using QPCR. For each colony, a microscopy image sequence showing colony formation is quantified using an image recognition tool in CL-Quant (Nikon). Our hypothesis is that live cell imaging metrics can be used to score colonies in terms of their differentiation bias. Here we present the results from these 40 cell lines together with the image-based measurements that correlate with cardiomyocyte outcomes.

Poster Board Number: T-3282

XENO-FREE CULTURE SYSTEMS FOR PLURIPOTENT STEM CELLS

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In recent years, there has been an increasing demand for qualified media and reagents that are free of animal derived ingredients for culturing pluripotent stem cells (PSCs). An important consideration for PSCs to be cultured in defined feeder-free and xeno-free conditions is that animal origin products may increase the risk of non-human pathogen transmission and immune rejection, limiting their use in downstream applications. To overcome such issues, we developed and tested a set of xeno-free reagents for PSC culture. The CTS™ culture system enables a complete and comprehensive solution for successful culturing of hESCs and iPSCs in KnockOut™ SR XenoFree CTS™ (KSR XF) (culture medium) and KnockOut™SR Growth Factor Cocktail (supplement) with CELLstartTM CTSTM (xeno-free matrix) that meet cell therapy compliant requirements and regulations. Cells grown in these conditions express normal pluripotent markers, maintain normal karyotype, and retain their ability to differentiate into cells of the three germ layers. Additionally, we also successfully derived iPSCs under xeno-free conditions using a non-integrating CytoTune™ -iPS Sendai Reprogramming Kit, and expanded the iPSCs under xeno-free conditions. This xeno free system will help researchers to transition towards translational and clinical research for PSC. CTS™ regulatory-compliant reagents such as KSR XF, GFC supplement and CELLStart™, will facilitate standardization and optimization of cell culture processing, minimize exposure of PSC lines to non-human animal origin material and render safety in cell therapy applications.

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Poster Board Number: T-3283

DIFFERENCE IN GLOBAL GENE EXPRESSION PROFILE AND STABILITY BETWEEN ES AND IPS CELL-DERIVED NEURAL STEM CELLS

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Induced pluripotent stem (iPS) cells, which were reprogrammed from somatic cells by overexpressing reprogramming factors, undergo unlimited self-renewal and have differentiation potential into various types of cells like embryonic stem cells (ESCs). Direct differentiation into a specialized cell types from iPS cells hold considerable promise for regenerative medicine as well as basic research. Neural stem cells (NSCs) are self-renewing multipotent populations and have capacity of neural differentiation into multiple cell lineages such as neurons, astrocytes, and oligodendrocytes. Many researchers have reported that NSCs have therapeutic effects in neurological disease following transplantation. Here, we induced differentiation of iPS cells into NSCs in vitro and in vivo, which were compared with ESC-derive and brain-derived NSCs. NSCs differentiated from ESCs and iPS cells were morphologically indistinguishable from brain-derived NSCs and rapidly propagated in the presence of EGF and bFGF, and stained positive for NSCs markers Nestin and Sox2. Moreover, these iPS cell-derived NSCs showed the tripotent capacity of differentiation into neurons, astrocytes, and oligodendrocytes. However, global gene expression pattern of iPS cell-derived NSCs (both in vivo- and in vitro-derived) were distinct from the brain-derived NSCs. Moreover, iPS cell-derived NSCs were spontaneously aggregated during the long-term passaging and formed ES cell like colonies, which finally expressed Oct4-GFP. The spontaneously reverted GFP-positive cells (iPS-NSC-iPS) expressed similar levels of pluripotency markers (Oct4, Nanog) to ESCs and iPS cells, and were able to form germline chimera. This re-reprogramming may be due to spontaneous re-activation of transgenes inserted in iPS cell genome when iPS cells were differentiated into NSCs. Taken together, iPS derived NSCs were morphologically and characteristically similar to brain-derived NSCs, but differ in global gene expression pattern and tend to reverted into pluripotent state spontaneously.

Poster Board Number: T-3284

ULTRASTRUCTURE OF PORCINE INDUCED PLURIPOTENT STEM CELL-LIKE COLONIES AND DERIVED EMBRYOID BODIES

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The generation of induced pluripotent stem cells (iPSCs) from terminally differentiated cells provides exciting possibilities for designing patient-specific *in vitro* disease models and producing immune-compatible tissues for transplantation. IPSCs have been produced from both mouse and human, however, the derivation of bona fide iPSCs from pigs is still pending. Porcine iPSCs would be extremely useful, since autologous transplantation in porcine biomedical models could serve as an intermediate step between mouse and man. The present study aimed to characterize the ultrastructure of porcine iPSC-like colonies reprogrammed from porcine neural progenitor cells (NPCs) by lentiviral transduction with a polycistronic construct encoding the transcription factors OCT4, SOX2, KLF4, and C-MYC. Two cell lines with embryonic stem cell (ESC)-like morphology, capable of prolonged clonal propagation, were analyzed at passage 24 as well as after embryoid body (EB) formation at this passage. Transmission electron microscopy was applied to look for stem cell characteristics and signs of differentiation. The iPSC-like colonies consisted of naïve cells with large ovoid nuclei containing abundant euchromatin and, typically, one to two distinct nucleoli. The cytoplasm contained lipid droplets, free ribosomes, and small rounded mitochondria with few cristae as the most prominent structures. Additionally, many cells had well-developed rough endoplasmic reticulum. Microvilli were commonly protruding between cells and at the borders of colonies, and the basal body and axoneme of a cilium were also found. Apoptosis was occasionally seen at the periphery of colonies, and phagocytosed apoptotic bodies were present in the cytoplasm of some cells. On one occasion, columnar epithelial differentiation had occured at the colony surface, where adjacent cells were linked with tight junctions and desmosomes. Overall, these observations are indicative of an undifferentiated ESC-like morphology of the porcine iPSC-like colonies, although apoptosis and signs of spontaneous differentiation were also noted. EBs produced from the iPSC-like colonies presented cells with a radically altered ultrastructure. The nuclei were extensively lobulated and contained electron-dense blocks of heterochromatin. The cytoplasm was rich in organelles; especially the smooth endoplasmatic reticulum and Golgi compartments were abundant, and intermediate filaments and microtubules composed a meshwork throughout the cells. Tight junctions and desmosomes were commonly found in the EB periphery. Furthermore, mitoses were evident. In two cases, neural rosettes consisting of radially arranged columnar cells, linked with tight junctions and desmosomes and enclosing a central cavity into which abundant cilia protruded, were observed. No other morphologically distinct cell types were detectable in the EBs, underscoring the inherent capability of the NPC-derived iPSCs for neural differentiation. It is tempting to speculate that this phenomenon may be due to an epigenetic memory of the NPCs potentially retained through incomplete iPSC reprogramming. In conclusion, porcine NPC-derived iPSC-like cells have an undifferentiated ultrastructure compatible with the potential state of pluripotency, whereas EBs derived from them revealed an inherent capability of neural differentiation.

Poster Board Number: T-3285

PIG IPS CELLS-A NEW RESOURCE TO GENERATE GENE MODIFIED PIGS

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Pig is one of the best organ donors to provide organs for patients because the porcine organs are significantly similar to humans in its dimension, structure and function, and serve as a research model in many fields of biomedical research. However, the number of transgenic pigs which have been reported is extremely limited, and even fewer knockout pigs have been generated by somatic cell nuclear transfer (SCNT) due to the lacking of pluripotent stem cells as the vector to deliver gene modification into the genome. Embryonic stem (ES) cells are the best cells for gene targeting to create transgenic animals, which has been demonstrated in the mouse. Although numerous attempts were undertaken to establish the pluripotent ES or EG cell lines, there was no validated pig ESC lines established to date. The iPS (induced pluripotent stem cells) technique provides a feasible approach to establish pluripotent stem cells for the species in which ES cell lines have been proven to be difficult to establish from the early embryos. Here we generated

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pig iPS cells using the viral system that expressed defined transcription factors. These iPS cell lines express the correct pluripotency markers, show normal karyotypes and high levels of unmethylation and telomerase activity, and can differentiate into all three germ layers both in embryoid bodies and teratomas. The next step to fulfill the promise of pig iPS cells is to generate a live pig from these cells. We are making progress to generate cloned pigs from pig iPS cells. Our findings about pig iPS cells should represent significant and interesting development that carries important potential implications for cell biology, medical science and agriculture.

Poster Board Number: T-3286

GENERATION OF INDUCED PLURIPOTENT STEM CELLS FROM BOVINE TESTICULAR CELLS WITH ONLY HOMEODOMAIN TRANCRIPTION FCTOR OCT4

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Pluripotent embryonic stem cells (ESCs) from domestic animals are useful for the production of genetically modified livestock and hold great promise for cell or organ therapies, drug screening, and the generation of human disease models. Although many attempts have been made to establish ESCs from large domestic animals, teratoma formation in all three germ layers has not been confirmed. We report here the first generation of bovine induced pluripotent stem cells (iPSCs), using transfection by electroporation of a single gene encoding a defined factor of homeodomain transcription factor 4 (OCT4). Supplementation with leukemia inhibitory factor and bone morphogenetic protein 4 maintained and stabilized the expression of ESC-specific genes by the bovine iPSCs and their differentiation potency. The bovine iPSCs displayed the normal karyotype and expressed alkaline phosphatase, SSEA-1, SSEA-4, OCT4, NANOG, c-MYC, KLF4, SOX2, STAT3, DNMT1, SUZ12, and MEF2A. These cells could also differentiate into cell types of all three germ layers in teratoma. The results of our study indicate that bovine iPSCs make the use of cattle in targeted experiments, feasible, to improve their genetic traits, their disease resistance, and their production of pharmaceutical proteins.

Poster Board Number: T-3287

GENERATION OF NAÏVE PORCINE INDUCED PLURIPOTENT STEM CELLS CAPABLE OF CONTRIBUTING TO EMBRYONIC AND FETAL DEVELOPMENT

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Pluripotent stem cells (PSCs) are of 2 types_naïve and primed. Ontogenetically, naïve PSCs correspond to preimplant inner cell mass (ICM) cells of blastocysts and primed PSCs, to postimplant epiblasts. Most striking difference between the two types is their capacity to develop offspring chimeras. Naïve PSCs have it, while primed PSCs have very limited one. Mouse PSCs are naïve and human PSCs are primed. Notably, human embryonic stem cells are derived from preimplant ICM, but they do not become naïve but become primed ones from unknown reasons. When you make PSCs from animals (monkeys, pigs, rabbits, etc) other than mice by the conventional method, you will obtain primed ones which are similar to human PSCs from unknown reasons, too. Therefore, it is difficult to utilize these animal PSCs for the purpose of developmental engineering; ie, in order to generate chimeric and knockout animals. It is required to obtain naïve PSCs to generate genetically modified animals from PSCs. In addition, compared to primed PSCs, naïve PSCs grow more rapidly, and are easier for the passage, freeze-and-thaw, genetic transduction, homologous recombination, and subcloning. Thus, naïve PSCs have clear advantages over primed ones. Can naïve animal PSCs be generated? We have tried to generate naïve porcine induced pluripotent stem cells (iPSCs). Porcine embryonic fibroblasts were transduced with Yamanaka's four genes by retroviral vectors. The cells were cultured under some modified conditions. Emergent colonies were round-form, which were similar to those of mouse PSCs. The cells typically expressed pluripotency markers and developed teratomas in immunodeficient mice, and thus they were iPSCs. The naïve state of porcine iPSCs was demonstrated by LIF-dependency, negative MHC class I, and two active X chromosomes (XaXa) when female, and characteristic gene expression profiles. In addition, the porcine iPSCs grow as rapidly as mouse PSCs. They can be easily passaged without a ROCK inhibitor. Thus, the porcine iPSCs are easy to handle, just like naïve mouse PSCs. Most importantly, when injected into the morulae, the porcine iPSCs contributed to the in vitro embryonic development to the blastocysts (11/24, 45.8%). They also contributed to the *in utero* fetal development (11/71, 15.5% at day 23; 1/13, 7.7% at day 63). Currently, we are trying to generate offspring chimeras.

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GENERATION OF INDUCED PLURIPOTENT STEM CELLS FROM TRANSGENIC PIG PRODUCED FOR XENOTRASPLANTATION

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The generation of embryonic stem cell (ESC) and induced pluripotent stem cell (iPSC) has great potential for the application of stem cell technology. However, many barriers are in the usage of human ESC or iPSC as a source of cell therapy, especially a major obstacle is no means to test the efficacy and safety of the therapy. The generation and application of porcine iPSCs (piPSCs) as a large animal model may be one of strategies to overcome these barriers in human regenerative medicine. Here, to our knowledge we report for the first time the generation of piPSC from genetically modified pig, alpha1,3-Galactosyltransferase knock-out (-/-) (GalT KO) and CD46 (membrane cofactor protein) knock-in (CD46 KI). Fibroblasts were isolated from the ear skin of a 10-day-old NIH miniature pig (GalT KO/CD46 KI). After 1 or 2 passage, fibroblasts were transduced with cocktail of 6 human factors (POU5F1, NANOG, SOX2, C-MYC, KLF4, and LIN28) and cultured on a mitotically inactive mouse embryonic fibroblast (MEF) monolayer. Reprogrammed somatic cells expressed the classical pluripotency markers (POU5F1, NANOG, SOX2, and SSEA1). Similar to mouse ESCs, piPSCs were negative for SSEA3, Tra-1-60, and Tra-1-81. Further these cells could form embryoid body (EB) and differentiate into 3 germ layers *in vitro*. Our piPSCs may provide useful source as a large animal model for studying approaches that can reduce an immune-rejection of cell or organ transplantation.

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IPS CELL REPROGRAMMING FROM FIBROBLASTS IS INDEPENDENT OF PIWI PROTEINS

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Piwi proteins function in the regulation of stem cell function, germline specification, transposon silencing, and the maintenance of genomic integrity across animal phylogeny. They bind to Piwi-interacting RNAs (piRNAs) and act as regulators of epigenetic programming. In addition, work in *Drosophila* shows that Piwi promote developmental robustness via the suppression of phenotypic variation. Given the pivotal role of Piwi/piRNAs in epigenetic modulation, we investigated the potential role(s) of Piwi proteins in induced pluripotent stem (iPS) cell reprogramming. The mouse genome encodes three Piwi homologs; Miwi, Mili, and Miwi2. We show that all three mouse Piwi genes are expressed in embryonic stem cells at significantly higher levels than in fibroblasts, with Mili being the highest. However, mouse embryonic fibroblasts derived from triple knockout (lacking Miwi/Mili/Miwi2) embryos were able to form as many iPS cell colonies as wildtype control cells. Furthermore, these cells expressed pluripotency markers and were capable of differentiating into the three germ layers in teratoma assays. Our results indicate that Piwi proteins are dispensable for direct reprogramming of mouse fibroblasts.

Poster Board Number: T-3290

MITOCHONDRIAL DYSFUNCTION AND ALFA SYNUCLEIN ACCUMULATION IN PARK2 IPSC DERIVED NEURONS AND POSTMORTEM BRAIN

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Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease in the world. Typical PD patients show abnormal motor functions, collectively called "parkinsonism", due to the loss of dopaminergic neurons in the substantia nigra. Recent discoveries of genes linked to familial PD have clarified pathogenesis of autosomal recessive juvenile PD (PARK2) with mutations in *parkin* gene, encoding an E3 ubiquitin ligase which is likely to be involved in the regulation of mitochondrial homeostasis. Although several animal or cell models with altered expression of *parkin* have been reported, it remains unclear whether these models actually recapitulate early cellular changes in the neurons of PD patient's. Here, we generated induced pluripotent stem cells (iPSCs) from two PARK2 patients, using retroviral vectors carrying four reprogramming factors, *Oct4, Sox2, Klf4* and *c-Myc*. Ten independent clones (3 from control and 7 from PARK2) with similar characteristics as human embryonic stem cells (hESCs). We confirmed that the iPSCs expressed pluripotent hESC markers and could form teratomas containing all three germ layers. All retroviral transgenes were silenced in each clone. The iPSCs from two PARK2 patients retained their homozygous *parkin* deletions and showed genome stability. The clones could all differentiate into neurons, including tyrosine hydroxylase (TH)-positive neurons, via embryoid body and neurosphere formation. The PARK2 iPSC-derived neurons showed abnormal metabolism and impaired mitochondrial homeostasis. Although PARK2 patients rarely exhibit Lewy body (LB) formation with α-synuclein accumulation, α-synuclein accumulation was detected in iPSC-derived neurons from one PARK2 patient. The accumulation observed *in vitro* was confirmed in the postmortem brain of the same patient. This is the first report to demonstrate pathogenic changes in the brain of PARK2 patients using iPSC technology. Thus, these phenotypes will provide a key to understand pathogenesis of PD and a potential therapeutic target.

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OVEREXPRESSION OF PGC-1 BY ADENOVIRAL VECTOR PROMOTED ADIPOGENIC DIFFERENTIATION IN INDUCED PLURIPOTENT STEM CELLS

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Induced pluripotent stem cells (iPSCs) are novel stem cell populations induced from mouse and human adult somatic cells through reprogramming by transduction of defined transcription factors. Peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α) plays an important role during brown fat development. However, the potential roles of PGC-1α in regulating mitochondrial biogenesis and the differentiation of iPSCs are still unclear. Here, we investigated the effects of adenovirus-mediated PGC-1α overexpression in iPSCs. PGC-1α overexpression resulted in increased mitochondrial mass, reactive oxygen species production, and oxygen consumption. Microarray-based bioinformatics showed that the gene expression pattern of PGC-1α-overexpressing iPSCs resembled the expression pattern observed in adipocytes. Furthermore, PGC-1α overexpression enhanced adipogenic differentiation and the expression of several brown fat markers, including uncoupling protein-1, cytochrome C, and nuclear respiratory factor-1, whereas it inhibited the expression of the white fat marker uncoupling protein-2. Furthermore, PGC-1α overexpression significantly suppressed osteogenic differentiation. These data demonstrate that PGC-1α directs the differentiation of iPSCs into adipocyte-like cells with features of brown fat cells. In addition, these results suggest that PGC-1α enhances adipogenesis but prevents osteogenesis.

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GENERATION OF INTEGRATION-FREE INDUCED PLURIPOTENT STEM CELLS FROM ADULT COMMON MARMOSET FIBROBLAST

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Transgenic non-human primate models for Human disease are available only in the common marmoset (*Callithrix jacchus*). In this species, the embryonic stem cells (ESC) and the induced pluripotent stem cells (iPSC) have been already established. They are useful for preclinical non-human models on regenerative medicine including xeno /auto graft. Although we have been generated marmoset iPSC from fetal liver cells, other tissue have not been generated iPSC. As the control for preclinical study of iPSC therapy, it is important that generation of integration-free iPSC from adult marmoset tissues that can be collected by low invasive method to perform xeno /auto graft. In this study, we perform the generation of integration-free iPSC from adult marmoset tissues using the sendai virus vector. The sendai virus (SeV) vector which has a single-strand RNA and is not integrated into the host genome was used to generate marmoset iPSC. At first, the cultured adult marmoset fibroblast was induced SeV coding green fluorescent protein (GFP) as reporter gene, and it was confirm that GFP expression in the marmoset fibroblast and positive rate depended on multiplicity of infection (MOI). Then, the marmoset fibroblast was infected temperature-sensitive SeV vector coding Oct3/4, Klf4, Sox2 and c-Myc. On day6, the inducted cells were replaced onto feeder layers. After 3-4 weeks of the induction, several colonies resembling marmoset ESC were shown and these colonies indicated alkaline phosphatase activity. Furthermore, the expressions of endogenous pluripotent marker genes were confirmed in these colonies by RT-PCR. These results indicated that the adult marmoset fibroblasts are able to reprogram to iPSC by SeV as well as fetal liver cells. This method would contribute in preclinical non-human primate model study on regenerative medicine.

Poster Board Number: T-3293

GENERATION OF IPSC FROM ADULT PROLIFERATING CNS PROGENITOR CELLS.

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Purpose. We have recently showed that RPE (retinal pigment epithelium) cells can be isolated from adult cadaver donor eyes and grown to form a functional epithelial monolayer. Furthermore, we have recently shown that the human RPE is a plastic cell with inducible stem-like properties. In this study, the adult RPE, which is also the only proliferative CNS cell that can be readily obtained by minor surgery, is used as a unique tissue source for iPSC production. Because the RPE is the disease-affected tissue in Age-Related Macular Degeneration (AMD), the leading cause of blindness in people over 50, there is good reason to think that RPE will produce a superior iPSC-based model of AMD. Methods. We routinely isolate adult RPE from cadaver donor eyes of different ages and sex. iPSC were derived from human adult RPE using a Sendai virus-based reprogramming strategy and characterized according to standard protocols, including immunostaining, qPCR and *in vitro* differentiation into the three germ layers. Results. Adult human RPE were successfully used as donor tissue to derive iPSC expressing pluripotency markers, including Tra-1-60, Nanog and SSEA4, and able to give rise to the three germ layers *in vitro*. No residual donor RPE cells were detected in the iPSC cultures. Conclusions. We were able to obtain iPSC from elderly donor using a mix of 4 factors, demonstrating that we can successfully reprogram RPE from donors with age ranging from 56 to 91 year old. To our knowledge, these are the first adult human CNS-derived iPSC, and the collection is particularly valuable as it includes elderly donors. In contrast to other sources of CNS stem cells in the hippocampal dentate gyrus and the subventricular zone, the RPE can be obtained relatively easily from live patients with minimal surgery, or from donor cadaver eyes. RPE-derived iPSC could be used to reveal the molecular pathways involved with aging and that underlie AMD. The essentially unlimited iPSC supply makes large-scale studies and drugs screens possible.

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PRE-EVALUATED SAFE HUMAN IPS CLONE DERIVED NEURAL STEM CELLS PROMOTED FUNCTIONAL RECOVERY AFTER SPINAL CORD INJURY WITHOUT TUMORIGENECITY IN ADULT COMMON MARMOSETS

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Introduction: Recently, we have reported the effectiveness of transplantation of mouse as well as human iPS derived neural stem cells (hiPSC-NSs) for spinal cord injury (SCI) in rodents. From the viewpoint of a clinical trial, it is critical to determine the effectiveness of human iPSC-NS transplantation in non-human SCI model. The purpose of this study is to determine the effectiveness and safeness of transplantation of human iPSC-NSs into the injured spinal cord of common marmosets. Methods: We used a pre-evaluated safe human iPS clone (hiPSCs), and neural differentiation of hiPSCs were induced through embryoid body using original protocol. A moderate contusive SCI was induced at the C5 level in adult common marmosets. 9 days after the injury, hiPSC-NSs at a density of 1x106cells/5ul were transplanted into the injured spinal cord. Behavioral analyses were performed according to the previous reports (original open field scoring scale, bar grip test, cage climbing test) until 12 weeks after SCI. Axial sections of spinal cords were subjected for the histological analyses to determine the effects of transplantation. Results: Grafted human iPSC-NSs that were survived and differentiated into NeuN positive neurons, GFAP positive astrocytes and Olig1 positive oligodendrocyte progenitor cells. It was noteworthy there was no tumor formation at least for 12 weeks after transplantation. Luxol fast blue and eriochrome cyanine stainings showed a significant decrease in demyelinated areas at the lesion epicenter in the transplanted group compared to the vehicle control group. Furthermore, quantitative analyses revealed that there were significant differences in CaMK2-alpha and RT97 positive areas at the epicenter between the transplanted and vehicle control groups. Quantitative RT-PCR revealed the expression of the vascular endothelial growth factor (VEGF), which is the source of the angiogenic signals, were significantly higher in the hiPSC-NSs than the human dermal fibroblast (hDF) *in vitro*. Consistent with the results of RT-PCR, there were significantly more platelet endothelial cell adhesion molecule-1 (PECAM-1) positive blood vessels at the lesion epicenter in the hiPSC-NSs transplanted group than in the vehicle control group. All of the examined behavioral tests also showed that transplantation if human iPS-NSs promoted the functional recovery after SCI in adult common marmosets. Conclusion: Grafted human iPS-NSs safely survived and differentiated into neurons, astrocytes, and oligodendrocytes without any tumorigenicity, thereby promoting functional recovery after SCI. Our results suggest that pre-evaluated safe hiPSC-NS therapy has major potential for the treatment of SCI in the clinical setting.

Poster Board Number: T-3295

DIFFERENTIAL METABOLIC BEHAVIOR SUPPORTED BY REMODELED SOMATIC METABOPROTEOME ENABLES INDUCTION OF PLURIPOTENCY

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Nuclear reprogramming successfully redirects somatic cells to the pluripotent state. Beyond genetic and epigenetic regulators of cell fate engagement, recent studies have implicated modulation of mitochondrial dynamics and energy metabolism in the execution of nuclear reprogramming events. Profiling the metabolome of murine somatic tissue versus induced pluripotent stem cells (iPS) demonstrated nuclear reprogramming-associated promotion of glucose metabolism, realized through greater utilization of glucose and accumulation of glycolytic endproducts. Real-time assessment unmasked downregulated mitochondrial reserve capacity and ATP turnover correlating with pluripotent induction success. Reduction in oxygen consumption and acceleration of extracellular acidification rates delineate a high throughput marker of the oxidative metabolism transition to glycolysis underlying programmed stemness acquisition. The bioenergetic switch was supported by a remodeled proteome, whereby 451 proteins were demonstrated altered through comparative proteomics of parental somatic fibroblasts and derived iPS cells. Mapped protein-protein relationships revealed prioritization of significantly overrepresented canonical pathways predicting differential metabolic behavior complementing architectural and signaling realignment in response to reprogramming stimuli. Assessment of network-associated biological processes pinpointed distinct metabolic reorganization. Specifically, upregulated processes included glycolysis, purine metabolism, biopolymer and macromolecular catabolism, while downregulated processes included regulation of phosphorylation, oxidative phosphorylation, amino acid metabolism and nucleotide biosynthesis. Altered protein expression and *in silico* categorization prioritized the impact of energy metabolism within the hierarchy of nuclear reprogramming development. Thus functional and structural metabolic reprogramming is integral to the induction of pluripotency, expanding on the genetic and epigenetic requirements for cell fate manipulation.

Poster Board Number: T-3296

DISSECTING TEMPORAL EVENTS DURING INDUCED REPROGRAMMING TO PLURIPOTENCY

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Reprogramming somatic cells to pluripotency through ectopic expression of transcription factors (Oct3/4, Sox2, Klf4, and c-Myc; OSKM) is a profound technology of which relatively little is known mechanistically. Induced pluripotent stem cell (iPSC) lines carrying drug-regulated transgenes are useful for tracing endogenous gene expression and epigenetic changes that occur during reprogramming, which guide subsequent cell-state transitions. We have developed single-copy, doxycycline (dox)-inducible, combinatorial transgene systems for reprogramming mouse somatic cells. Short-term induction of OSKM factors *in vivo* causes extensive, yet reversible, cellular dysplasia of epithelial tissues, while long-term treatment of chimeric mice results in tumours or fully differentiated

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teratomas. Dox-induction in in vitro cultured somatic cells, including mouse embryonic fibroblasts (MEFs), generates bona fide iPSCs, however, the kinetics, efficiency, and quality of generation correlates with the design of the transgene encoding the reprogramming factors. Differences in early transgene and protein expression levels manifest as disparate effects on cell proliferation, morphology, and colony formation, presumably through modulation of the mesenchymal-epithelial transition (MET). Late-stage expression levels impact endogenous pluripotency gene expression and iPSC quality. We are now employing these composite systems in MEFs to reveal direct effects on transcriptional regulatory networks mediated by the reprogramming factors. Our data implies discrete reprogramming factor requirements at each stage of the process, and prospective refinement of current reprogramming standards.

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EFFICIENT METHOD TO GENERATE VECTOR-FREE INDUCED PLURIPOTENT STEM CELLS FROM CD34+ AND PBMCS USING THE CYTOTUNE™ -IPS SENDAI REPROGRAMMING KIT

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The most common source of human cells for the generation of induced pluripotent stem cells (iPSCs) have been fibroblasts. However, fibroblasts are obtained from skin biopsies which require invasive surgical procedures and limit the ability to generate scalable iPSCs from existing tissue sources. While peripheral blood mononuclear cells (PBMCs) and CD34+ can be readily obtained from blood, existing methods encounter technical challenges to convert peripheral blood cells to iPSCs consistently and with high efficiency. In addition, the conventional methods use integrating virus or plasmid based method to reprogram, which could potentially result in multiple insertions and risk of tumorigenicity. Here we demonstrate a highly efficient method to reprogram PBMCs or CD34+ to iPSCs using the CytoTune™ -iPS Sendai Reprogramming Kit which utilizes non-integrating Sendai virus vectors. The iPSCs were characterized and showed absence of trans-genes and viral vector by real-time TaqMan® Assays. The iPSC clones express proper pluripotent markers and differentiate into three lineages. These studies demonstrate an easy to use method to generate iPSCs from peripheral blood and facilitate the transition to high production of iPSCs.

Poster Board Number: T-3298

IDENTIFICATION OF TRANSCRIPTION FACTORS INVOLVED IN THE REPROGRAMMING PROCESS

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One of the most important roles of transcription factors is to regulate cell fate decision. In many cases, however, how transcription factors work to decide the cell fate is veiled in mystery. The reprogramming process from mouse embryonic fibroblasts (MEFs) to induced pluripotent stem cells (iPS cells) also remains incompletely understood. Our goal is to identify reprogramming specific changes of gene expression and their master regulators. Our microarray analysis identified 532 genes whose expression levels showed more than two fold upregulation in the reprogramming process, and these genes were classified into three groups according to their expression patterns. Then we attempted to predict transcription factors responsible for the gene expression changes in each group. Our promoter analyses and the subsequent screening processes identified two candidates, Foxd1 and Foxo1. Foxd1 was previously shown to be required for the development of retina and the morphogenesis of kidney. In the reprogramming process, downregulation of Foxd1 by RNA interference decreased the number of iPS cell colonies, and reduced expression levels of the predicted downstream target genes of Foxd1. Another candidate Foxo1 was previously shown to be involved in several intracellular signaling pathways, and required for the maintenance of ES cell pluripotency. Downregulation of Foxo1 also resulted in the decreased reprogramming efficiency. Studies on how Foxd1 and Foxo1 work are in progress.

Poster Board Number: T-3299

ESTABLISHMENT AND FUNCTIONAL ANALYSIS OF IPS CELLS WITH DRUG-INDUCIBLE SYT-SSX FUSION GENE

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Purpose: Synovial sarcoma (SS) is a malignant soft tissue tumor harboring a tumor-specific fusion gene, SYT-SSX, which consists of SYT (at 18q11) and either SSX1 or SSX2 (both at Xp11). Based on the high sensitivity and specificity of this fusion gene, the fusion product is deeply related to tumorigenesis of SS. SYT-SSX chimeric proteins have been reported to regulate the transcription of specific target genes, although the roles are not clearly known. Forced expression of SYT-SSX gene induced cell death in normal cells except Rat1, which was successfully transformed into tumorigenic cells. These results suggest that only cells on particular lineages can be permissive for SYT-SSX fusion protein. To identify such cell-lineage, we established human induced pluripotent stem cells (iPSCs) containing drug-inducible SYT-SSX fusion gene, which can differentiate into every type of human cells. Materials and Methods: Human iPS cells established either by retroviral or plasmid vectors were used as parental cells. PiggyBac transposon system with Tet-ON construct was used to improve the integration efficiency. After cloning SYT-SSX fusion gene into Tet-ON construct, PiggyBac vector was co-transfected with a vector expressing transposase gene. Appropriate clones were selected by the response after doxycyclin treatment. Results: We succeeded to establish iPSCs with inducible SYT-SSX that showed minimum leaky expression and efficient Dox-concentration-dependent induction of SYT-SSX fusion gene. The morphology of such iPS cells was indistinguishable from that of parental cells, whereas induction of SYT-SSX fusion gene dramatically changed their morphology within 48 hours after induction. Expression of stem cell marker genes was also significantly inhibited by SYT-SSX fusion gene. Conclusion: Human iPS cells with drug-inducible SYT-SSX fusion gene were successfully established. This system will be a useful tool to analyze the role of SYT-SSX fusion protein in tumorigenesis and also the cell-of-origin of SS.

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LARGE-SCALE AND FOCUSED TRANSCRIPTOME ANALYSIS OF INTEGRATION-FREE INDUCED PLURIPOTENT STEM CELLS

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Pluripotent stem cells such as embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC) are commonly identified and characterized based on biomarker expression. While commonly used surface marker expression provides a method for screening and identifying emerging iPSC clones for further expansion, detailed characterization of the established iPSC clones is necessary before its use for downstream applications. Several platforms are available for gene expression analysis varying in content and complexity. To determine the optimal method and minimal set of genes required for definitive characterization of pluripotency, iPSCs were generated using the integration-free CytoTune™- iPS Sendai Reprogramming kit. Established clones were expanded on feeder-based and feeder-free culture conditions and confirmed to have normal karyotype, express pluripotent surface markers and possess Tri-lineage differentiation potential. These clones were included in detailed transcriptome analysis using medium density TaqMan® OpenArray® qPCR platform and Illumina whole genome array. Some samples were further characterized by Next Generation sequencing RNA Seq using Ion PGM™ Sequencer. Analysis of the data indicates that iPSCs generated using the CytoTune™-iPS Sendai Reprogramming kit are similar to a control iPSC line generated using non integrational episomal vectors and H9 ESC, but distinct differences was observed between feeder-dependent and feeder-free iPSC lines. Such differences in gene expression were not observed in H9 ESC grown on feeder dependent vs. feeder free conditions. Functional analysis of differential expressed genes showed that genes related to DNA replication and cell cycle was more richly represented in feeder-dependent conditions than feeder-free lines. To determine if the difference was due partial reprogramming of the feeder-free systems, later-passage cells were analyzed. Comparison of massively parallel methods to identify novel markers and utilization of large scale arrays to confirm such markers paves way to distill the content into focused set of genes that can be validated on medium density qPCR OpenArray® fixed content panels for high through put analysis of multiple samples.

Poster Board Number: T-3301

INDUCED PLURIPOTENT STEM CELL AND ES CORE FACILITY OF THE ERASMUS STEM CELL INSTITUTE FOR REGENERATIVE MEDICINE

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The discovery of induced pluripotent stem cells (iPSC) in 2006 had an enormous impact on study of human disease and provides starting material in disease models for regenerative medicine and for drugs screening. Several methodologies have been published to generate iPSC lines, but the process is labor intensive and requires defined standard operating procedures to provide a consistent and reproducible outcome of the reprogramming process. The iPSC & ES core facility of Stem Cell institute at Erasmus medical center was established in 2010 with the mission to generate iPS cells from mouse & patient material to accelerate research in the stem cell field supporting the research groups at the Erasmus medical center and academic communities. We are currently using a Polycistronic lentiviral reprogramming construct including a fluorescent marker (dTomato) 1 for reprogramming human fibroblasts and four retroviral reprogramming factors2 to reprogram mouse fibroblasts. We have generated normal and disease specific iPSC lines using fibroblast cells obtained from skin biopsies of schizophrenia, fragile X patients, & patients with X-chromosome abnormalities. In addition, several mouse embryonic fibroblast cells representing different mouse models were reprogrammed. Our quality control involves characterization of our iPSC lines by morphology, cell surface markers, expression of pluripotent markers such as Nanog, Klf4, Sox2, Oct3/4, c-Myc, Fgf4, Rex1; their capacity to differentiate and karyotype & mycoplasma analysis. In mouse X chromosome reactivation happens in female cells concomitantly with the reprogramming process providing a convenient readout for pluripotency. In human this relationship is less clear. To establish the activity of both X chromosomes in our female iPS cell lines, we will use a combination of RNA-FISH analysis detecting XIST RNA, which associates with the inactive X, and immunohistochemistry detecting the histone modifications, H3K4me3 & H3K9acetyl, specific for the inactive X chromosome,. As a core facility, we aim to deliver state of the art iPS cells with the best available reprogramming protocols. We also facilitate the iPS research of customers with on side training of tissue culture practice, by providing tissue culture protocols, and the facilitation of the iPS related research. In near future, we aim to extend our service including new donor cell types such as blood and stromal cells. References: 1. Lentiviral Vector Design and Imaging Approaches to Visualize the Early Stages of Cellular Reprogramming. Molecular Therapy vol. 19 no. 4 apr. 2011 2. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126, 663-676.

Poster Board Number: T-3302

ACTIVATION OF INNATE IMMUNITY IS REQUIRED FOR EFFICIENT NUCLEAR REPROGRAMMING

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The generation of induced pluripotent stem cells (iPSCs) by Yamanaka and colleagues, first reported in 2006, was a seminal development in regenerative medicine. However, much remains to be understood about the underlying mechanisms of reprogramming of somatic cells to iPSCs. Consequently there is concern regarding potential clinical applications in the absence of mechanistic insights. Although retroviral overexpression of the reprogramming transcription factors (Oct4, Sox2, Klf4 and c-Myc) generates iPSCs, the integration of foreign DNA into the host genome could silence indispensable genes or induce genomic dysregulation. One approach to overcoming these limitations is to express the transcription factors as cell-permeant proteins, yet to date this has proved difficult. Moreover, human somatic cells have not been reprogrammed using purified cell permeant peptides (CPPs). In seeking to develop this alternative approach, we discovered a striking and consistent difference in the pattern of gene expression induced by viral versus protein-based delivery of the reprogramming factors. The difference in the pattern of gene expression suggested to us

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that a signaling pathway required for efficient nuclear reprogramming was activated by the retroviral, but not CPP approach. We suspected, and then confirmed, that the viral vector was more than a mere vehicle for genes encoding the reprogramming factors. In both gain- and loss-of function studies, we find that activation of toll-like receptor 3 (TLR3) plays a role in the efficiency of nuclear reprogramming. Stimulation of TLR3 causes rapid changes in the expression of epigenetic modifiers, with chromatin remodeling and changes in gene expression, which favor induction of pluripotency. These studies highlight the gaps that remain in our understanding of nuclear reprogramming, and for the first time suggest a collaborative contribution of inflammatory pathways in the induction of pluripotency.

Poster Board Number: T-3303

PATTERNED NEURAL AND CARDIAC DIFFERENTIATION OF ONE EB OF NANOG-IPS USING A MICROFLUIDIC DEVICE

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Embryonic stem (ES) or induced pluripotent stem (iPS) cells are widely applied in fundamental research because of their ability of self-renewal and differentiation into many derivatives. Regulation over two kinds of differentiation simultaneously in one EB (embryoid body) is necessary and important in fundamental and biomedical fields at present. So far, there are still no these kinds of reports because of some practical difficulties in mimicking *in vivo* surroundings. It is very difficult to imitate over two kinds of chemical gradients in micro-scale in vitro by only conventional methods of using dishes or plates, which play important roles in directing *in vivo* differentiation spatiotemporally. Some microfluidic devices had been fabricated for culturing and collecting stem cells, even for controlling simple differentiation. However, great physical shear or strain generated by flow would affect cellular proliferation or differentiation when chemical gradient was maintained continuously in space. Therefore, it is expected to regulate over two kinds of differentiation spatiotemporally by new methods (new devices). For the purpose, a microfluidic device was designed, which is composed of two flow channels, and an EB seeding channel located between the flow channels. An EB can be seeded and cultured at the channel crossing, where three PDMS pillars stand for preventing the EB from flowing away. The flow rate can be too small to affect the EB metabolism. At the same time, chemical gradients can be kept at the crossing point. The hypothesis was testified by theoretical simulation using COMSOL software. The theoretical simulation showed that the chemical gradient was kept at the crossing even when the flowing velocity was 0.1uL/min corresponding to the shear force of 0.07mPa, which is small enough to avoid its influence on the cells. After seeding an EB of mouse nanog-GFP iPS at the crossing, the flow channels were connected to syringe pumps, EB differentiation was induced by perfusing a medium containing FBS (20%) for cardiac differentiation and/or a medium containing N2 supplement for neural differentiation through the two flow channels respectively. After 4 days of culture, it was shown that dynamic culture in a microfluidic device promoted not only proliferation but also differentiation. It was also found that patterned differentiation was induced in one EB when the two kinds of media were perfused simultaneously. Immuno-staining experiments showed that a half of the EB derivatives was cardiac-troponin stained, interestingly, the other half was b3-tubulin stained. The experimental results of real time PCR indicated that gene expression of cardiac troponin and b3-tubulin was simultaneously observed in one EB. These results suggested that the chemical gradients for inducing cardiac and neural differentiation were kept at the crossing continuously and simultaneously, which effectively directed the two kinds of differentiation. The experimental methods of using the microfluidic device shows the advantages in maintaining a low level of shear, and keeping two chemical gradients simultaneously, which suggested that it is possible to control the patterned differentiation in one EB for future biomedical research.

Poster Board Number: T-3304

KDM2B ENHANCES IPS CELL GENERATION BY FACILITATING GENE ACTIVATION IN EARLY STAGE OF REPROGRAMMING

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Reprogramming from somatic cells to induced pluripotent stem (iPS) cells by transcription factors is by nature an epigenetic process of cell fate change. Previous studies have shown that the low reprogramming efficiency can be enhanced by introduction of additional factors. To investigate the mechanism of reprogramming, we aimed to identify epigenetic modulators promoting iPS cell generation. Here we report that Kdm2b, a histone H3 lysine 36 dimethyl (H3K36me2)-specific demethylase, promotes iPS cell generation. The capacity of Kdm2b to promote iPS cell generation depends on its demethylase activity and DNA binding property, but is independent of its role in regulating senescence and cell proliferation. We show that Kdm2b functions early in reprogramming and enhances the activation of early responsive genes by localizing onto and demethylating its target genes. Restricting the upregulation of early activated genes abrogates the capacity of Kdm2b in promoting reprogramming. Our studies not only identify Kdm2b an epigenetic factor important for iPS cell generation, but also uncover the mechanism of how Kdm2b contributes to the reprogramming process.

Poster Board Number: T-3305

NOVEL STRATEGY OF EPIGENETIC REPROGRAMMING WITH THE MYOD TRANSACTIVATION DOMAIN FOR IPS CELL GENERATION

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Induced pluripotent stem cells (iPSCs) can be created by reprogramming differentiated cells through introduction of defined genes, most commonly Oct4, Sox2, Klf4, and c-Myc (OSKM). A major difficulty of producing induced pluripotent stem cells (iPSCs) has been the low efficiency of reprogramming differentiated cells into pluripotent cells. Here we show that 30% of mouse embryonic fibroblasts (MEFs) were reprogrammed into iPSCs when they were transduced with a fusion gene composed of Oct4 and the transactivation domain of MyoD (called M3O), along with Sox2, Klf4 and c-Myc (SKM). Transduction of M3O and SKM into fibroblasts effectively remodeled patterns of DNA methylation, chromatin accessibility, histone modifications, and protein binding at pluripotency genes, raising the efficiency of making mouse and human iPSCs. In addition, M3O facilitated chromatin remodeling of pluripotency genes in the majority of transduced MEFs, including the cells that did not become iPSCs. These results identified that one of the most critical barriers to iPSC creation is poor chromatin accessibility and

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protein recruitment to pluripotency genes. The MyoD transactivation domain has a capability of overcoming this problem.

Poster Board Number: T-3306

EFFICIENT GENERATION OF INDUCED PLURIPOTENT STEM CELLS BY THE USE OF PTEN INHIBITOR

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The generation of induced pluripotent stem cells (iPSCs) that can differentiate into all cell types of the body like embryonic stem cells (ESCs) has brought us expectations for the human iPSCs to be used as a cell source for human regenerative medicine without any ethical problems. However, the inefficient generation of iPSCs (0.01â^¼0.1%) limited the utility of iPSCs. Previous studies have shown that Phosphoinositide 3-kinase (PI3K) pathway plays important roles in self-renewal and proliferation of embryonic stem cells (ESCs). The tumor suppressor, phosphatase and tensin homolog deleted on chromosome 10 (Pten), negatively regulates PI3K pathway and its inhibition leads to the activation of PI3K pathway. Recent reports have shown that suppression of Pten improves self-renewal, cell survival and proliferation of mouse and human ESCs. However the contributions of PI3K pathway or its negative regulator, Pten, to the process of reprogramming remain unknown. Here, we report that the activation of PI3K pathway by the inhibition of Pten, improves the reprogramming efficiency for iPSC generation. In this study, we retrovirally expressed four reprogramming factors Oct3/4, Klf4, Sox2 and c-Myc (OKSM) in Pten deficient mouse embryonic fibroblasts (MEFs). Round ESCs-like colonies were found around 7 to 10 days after the ectopic transduction of OKSM. To evaluate the efficiency of iPSC generation, we performed alkaline phosphatase (AP) staining assay. The number of AP+ colonies was significantly higher in OKSM- or OKS-transduced Pten-/- MEFs compared to that of controls, indicating that the efficiency of iPSC generation was significantly enhanced when Pten was deleted. We next activated PI3K pathway by the expression of phosphatase-deficient PTEN mutants (CS-Pten) or active myristoylated form of AKT (myr-AKT), and the efficiency of iPSC generation from wild-type MEFs by OKSM were examined. We observed significantly more AP+ colonies from MEFs expressing CS-Pten or myr-AKT compared to controls. These results indicate that the activation of PI3K pathway enhances the generation of iPSCs by the co-expression of OKSM. Continuous activation of PI3K pathway by the loss of Pten may lead to transformation of cells. Thus to reduce the risk of cell transformation, we transiently activated PI3K pathway by the use of Pten inhibitor, bpV(HOpic), during the reprogramming process for the generation of iPSCs (bpV-iPSCs) by the retroviral transduction of OKSM. The efficiency of iPSC generation was examined by AP activity. The number of AP+ colonies generated in the presence of bpV(HOpic) was twice higher than controls (approximately 9%). These bpV-iPSCs expressed immature cell markers such as SSEA1and Nanog, and showed a normal karyotype. Moreover, these bpV-iPSCs have the ability to differentiate into three-germ layers in vitro and *in vivo*. Furthermore, bpV-iPSCs contributed to germline-competent chimeric mice. These results indicated that bpV-iPSCs shared the similar pluripotency like ESCs. Notably we have also found that inhibition of Pten efficiently promoted the human iPSC generation. Overall activation of PI3K pathway by the use of Pten inhibitor, bpV(HOpic) dramatically improves the efficiency of iPSC generation from mouse and human somatic cells, and this technology may directly contribute to establish human regenerative medicine using iPSCs.

Poster Board Number: T-3307

GENERATION OF CANINE IPS CELLS AND THEIR CHARACTERISTICS

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For the clinical application of induced pluripotent stem cells (iPSCs) to re generative medicine, there are many issues to be overcome. One of them is to establish appropriate non-rodent animal models for preclinical experiments, in which the efficacy and the safety of the iPSCs-based treatments can be monitored. For this purpose, dog is a suitable animal because of its long life and anatomical and physiological similarities with human. In addition, do g and human share some pathological conditions with identical genetic cause such as muscular dystrophy and hemophilia. We are currently working on the generation of canine iPSCs from somatic cells to take those advantages. As the cell source for generating iPSCs, canine embryonic fibroblasts (CEF) were used. We have cloned canine Yamanaka's factors and other reprogramming factors, and constructed retroviral vectors expressing each factor. Two to three weeks after the introduction of these factors into CEF, several iPSC-like colonies appeared. Though those colonies showed iPSC-like morphology, few showed the expression of pluripotent markers such as Oct4/Sox2/Nanog in PCR. Therefore we have selected candidate colonies with puromycin selection by transducing selective construct of lentivirus vector and we have attempted a series of culture conditions. Finally we established an appropriate one for maintaining them with iPSC-like morphology even after 50 or more passages. Gene expression profiles and immunostaining of cell surface markers showed their characteristics which were basically similar to those of human pluripotent stem cells. We have also confirmed their differentiation property into all three germ layers in vitro, and are currently trying to confirm its differentiation property *in vivo*.

Poster Board Number: T-3308

REPROGRAMMING DEPENDING CD133 (PROMININ-1) EXPRESSION IN INDUCED PLURIPOTENT STEM CELLS

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CD133 (prominin-1) is expressed on different types of stem and progenitor cells originating from various sources, including the neural and hematopoietic systems. CD133 is used as a cell surface marker for somatic stem cell identification and isolation, but its physiological and biological functions of details are not yet well understood. Recent studies indicated that CD133 was not only expressed on bone marrow hematopoietic stem cells (SHCs), neural stem cells (NSCs), undifferentiated embryonic stem cells (ESCs) and differentiated ES cell progeny, but also expressed on several cancer cells such as lung or colon cancers. Although cell reprogramming can convert somatic cells to ESC-like cells that was named ¡¥induced pluripotent stem cells (iPSCs), by using direct transduction of a cocktail composed of only four pluripotent transcription factors: Oct4, Sox2, Klf4 and c-Myc, however this cell reprogramming to completed iPSCs would be processed from initiation to full repro-

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gramming pass though partially reprogrammed cells (pre-iPSCs). In order to study the role of CD133 during the processing of cell reprogramming, we analyzed CD133 expression of mouse iPSCs that were produced by retroviral transduction of defined four transcription factors in early stages of the pre-iPS cells, and later stages of the reprogramming processes after 10 passages of cultured iPSCs. In our immunofluorescent results, CD133 was shown significant expression in iPSCs after 10 passages of cultures than in the early stages of the pre-iPSCs. On other hand, CD133 expression also was shown markedly on mouse ESCs, but weakly on bone marrow-derived mesenchymal stromal cells and mouse embryonic fibroblasts (MEF). This result suggests that as well as changes in the expression of CD133 during the cell reprogramming processes from the partial reprogramming to full reprogramming for generation of iPSCs.

Poster Board Number: T-3309

REPROGRAMMING COCHLEAR CELLS INTO INDUCED PLURIPOTENT STEM CELLS WITH OCT3/4, SOX2 AND KLF4

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The loss of regenerative ability of hair cells in the mammalian contributes to the permanent sensorineural hearing loss. In embryonic or neonatal mammalian inner ear, cochlear stem/progenitor cell populations (otospheres) have been identified, while cochlear stem cells appear to diminish in number after birth and become quiescent in adult. Yamanaka's group has established the method for reprogramming of somatic cells into induced pluripotent stem (iPS) cells by introduction of transcription factors, the established iPS cells has provided great potential for tissue specific regeneration. The aims of present study were to examine whether transcription factors can induce reprogramming of otosphere cells, which may result in generation of iPS cells from cochlear cells. We introduced transcription factors, Oct3/4, Sox2 and Klf4 into otospheres derived from postnatal day-1 mouse cochlear eptihelia using retrovirus, and analysed alterations in cell characteristics. After transduction, otospheres generated colonies that displayed embryonic stem cell (ESc)-like morphology and expressed pluripotent markers of iPS cells. iPS cells derived from otospheres (OiPS cells) are able to differentiate into cells from three germ layers in vitro and contributed to the formation of teratoma *in vivo*. These findings demonstrate that transduction of transcription factors is capable of inducing reprogramming of otospheres, suggesting that the method for generation of iPS cells might be utilized for full or partial reprogramming of cochlear cells.

Poster Board Number: T-3310

GENERATION OF GERMLINE TRANSMISSION COMPETENT IPSCS IN 7 DAYS

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Somatic cells can be reprogrammed into induced pluripotent stem cells (iPSCs) by defined factors. However ,the efficiency is considerably low and the process is time-consuming. We first exploited a chemical defined medium, named iCD1, in which the MEFs can be reprogrammed with high efficiency and fast kinetics by Oct4/Sox2/Klf4. Utilizing blastocysts injection ,here we report that the iPSC generated at D7 post-infection in iCD1 has the ability to contribute to chimera mice and germline transmission. Thus ,we established a technical scheme to determine fully reprogramming ,and first proved that somatic cells can be fully reprogrammed in a 7 day procedure with only three reprogramming factors.

Pre-clinical and Clinical Applications of Mesenchymal Cells

Poster Board Number: T-3311

RECONSTRUCTION OF HUMAN ELASTIC CARTILAGE BY PROGENITOR CELLS IN THE EAR PERICHONDRIUM

Suzuki, Hiromu, Kobayashi, Shinji, Takebe, Takanori, Yabuki, Yuichiro, Mizuno, Mitsuru, Murata, Syunsuke, Tamura, Yuko, Yasumura, Kazunori, Maegawa, Jiro, Taniguchi, Hideki
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Introduction: Future cell-based therapies such as tissue engineering will benefit from a source of autologous cartilage progenitor cells. In human auricular elastic cartilage, the cartilage progenitor cells have not yet been identified. It is expected that the cells will have new possibilities for better cartilage reconstruction and be a useful cell resource in place of mature chondrocytes. The human auricular elastic cartilage consists of the chondrium, and the perichondrium that has been known for regenerating cartilage clinically. Therefore, it is presumed that the cartilage progenitor cells of auricular cartilage might exist in the perichondrium. The purpose of this study is to identify cartilage progenitor cells of human auricular elastic cartilage and utilize for cartilage reconstruction. Methods: The perichondrium, the interlayer, and the chondrium were harvested from pieces of retained auricular cartilage of microtia patients. Cells isolated from their tissues were defined as the perichondrocytes, the interlayer cells and the chondrocytes. To analyze the proliferative ability, clonogenicity assay and long-term cell growth assay were done. In order to characterize surface marker profiles, we performed flow cytometry. The hematopoietic stem cell markers, and the MSC markers were analyzed. The multipotency was tested in vitro chondrogenic, adipogenic and osteogenic differentiation assays. To study cartilage regeneration capacity in vivo, perichondrocytes and chondrocytes inducted cartilage differentiation in vitro were implanted in NOD/SCID mice and after 1, 3 months estimated histologically. Results: The perichondrocytes isolated from the perichondrium showed the highest proliferative ability of cells isolated from the others in both clonogenicity and long-term cell growth assay. Among the mesenchymal stem cell markers, CD44 and CD90 expressions showed significant differences. Higher expressions of CD44 and CD90 were observed in perichondrocytes compared with those of chondrocytes. The perichondrocytes expressed not only proteoglycan and collagen II but also lipid vacuoles and calcium by each induction mediums. Perichondrocytes and chondrocytes implanted in NOD/SCID mice reconstructed cartilage and their matrix was consisted of proteoglycan and collagen II histologically similarly. The perichondrocytes-derived cartilage has had perichodrium and the cell density not changed after 3 months, but the chondrocytes-derived cartilage not perichondrium and the density decreased. Conclusion: Our study suggests that the perichondrocytes might be cartilage progenitor cells, because they had not only higher proliferative capacity than the chondrocytes but also multipotency to differentiate into osteocytes and adipocytes. Surface marker profiles indicated that these progenitor cells were potentially enriched by combinations of CD44 and CD90. Moreover, we demonstrated that cultured human perichondrocytes could reconstruct cartilage in vivo and the perichondrocytes-derived cartilage might maintain the cartilaginous phenotype in the long term in comparison with chondrocytes. The utilization of progeni-

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tors from human auricular perichondrium will not only improve our understanding of basic cartilage biology, but will lead to novel therapeutic strategies, including long-term tissue restoration, for patients with craniofacial defects.

Poster Board Number: T-3312

TRANSPLANTATION OF HUMAN ADULT STEM CELLS PROTECTS AGAINST SOCIAL DEFICIT EXHIBITED BY SUB-CHRONICALLY PHENCYCLIDINE TREATED MICE

Barzilay, Ran, Ganz, Javier, Segal, Hadar, Ben-Zur, Tali, Bren, Ziv, Hinden, Noa, Tarasenko, Igor, Taler, Michal, Lev, Nirit, Sadan, Ofer, Gil-Ad, Irit, Weizman, Abraham, **Offen, Daniel**

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Background:Stem cell based regenerative therapy is a promising therapeutic approach, bringing hope for patients affected with incurable diseases. Mesenchymal stem cells (MSCs) represent an attractive cell source for regenerative medicine strategies for the treatment of brain diseases, exerting neuroprotection and inducing neurogenesis. Schizophrenia (SCZ) is a devastating brain disease with poor prognosis, especially due to the debilitating negative symptoms. Beside a neurodevelopmental element, the pathophysiology underlying SCZ involves neurodegeneration, oxidative stress and impaired neurogenesis. In the current study, we sought to explore the prospect of intracerebral MSCs transplantation for treating the phencyclidine (PCP) SCZ mouse model. PCP was administered subcutaneously to C57bl mice (10mg/kg daily for 2 weeks). Adult human MSCs transplantation into the hippocampus was conducted on the day of first PCP administration. Social preference test was conducted 10 days following the last PCP administration. Results: Transplantation resulted in a significant reduction in the impairment of social phenotype induced by the PCP insult, as observed by the social preference test. Immunohistochemical analysis revealed that the human cells survived in the mice brain throughout the course of the experiment (25 days). Western blot analysis of the hippocampus showed a decrease in cleaved caspase3 and an increased Bcl-2 protein levels in the brains transplanted with MSCs. Moreover, proteins involved in glutamate metabolism and oxidative stress which were affected by the PCP insult were positively regulated by the MSCs transplantation. Interestingly, co- culture of MSCs with mouse astrocytes in vitro showed that MSCs exert effect that counteracts changes in astrocytes gene expression following exposure to PCP. Conclusions: Delivery of MSCs to distinct brain regions, known to be involved in the pathophysiology of SCZ, is beneficial in attenuating the behavioral deficits exerted by the PCP insult in mice. We hereby suggest a novel therapeutic approach for the treatment of SCZ negative symptoms.

Poster Board Number: T-3314

DIFFERENTIATED MESENCHYMAL STEM CELL TRANSPLANTATION IN AMYOTROPHIC LATERAL SCLEROSIS (ALS) PATIENTS A PHASE I/II CLINICAL TRIAL

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Background: Amyotrophic Lateral Sclerosis (ALS) also known as Lou Gehrig's disease, is a fatal, rapidly progressive, neurodegenerative disease caused by motor neuron degeneration. Despite extensive efforts no treatment is currently available that slows, stops, or reverses disease progression. Bone marrow derived Mesenchymal stromal stem cells (MSC) represent an attractive cell source for the treatment of neurological diseases, exerting neuroprotection and possibly inducing neurogenesis. A previous pilot study from our group at Hadassah has shown the safety of the intravenous and intrathecal administration of unmodified MSC in ALS patients and provided some indications of clinical benefit in terms of stabilization of the disease. We have developed a protocol for MSC differentiation into cells that secrete neurotrophic factors (NTF), based on the Brainstorm's NurOwn™ technology. These MSC-NTF cells, demonstrated neuroprotective effects in various animal models of neurodegenerative diseases, including ALS. Aims: To evaluate the safety and tolerability of treatment with autologous MSC-NTF cells in ALS patients utilizing the intramuscular (IM) way of administration of the cells at the early stage of the disease and the intrathecal (IT) transplantation in patients with more advanced/progressive disease. Method/Design: This Phase I/II clinical study will include in total 24 ALS patients, assigned to 2 treatment groups based on the severity of their disease: 12 patients with early ALS (ALSFRS score of >30), and 12 patients with advanced ALS (ALSFRS score of 15-30). Patients from both groups are followed for three months prior to transplantation. Autologous bone-marrow derived MSC-NTF cells are transplanted, by IM or IT injections to patients with early or advanced ALS, respectively. After transplantation patients are followed up clinically (detailed neurological examination and ALSFRS evaluation) on a monthly basis for a post-treatment follow up period of 6 months. Respiratory function tests, MRI of the muscles and EMG will be used as additional surrogate markers of the disease activity. Results: Four ALS participants were recruited in the second half of 2011. MSC were isolated, expanded ex-vivo, purified and induced to differentiate into cells secreting neurotrophic factors (MSC-NTF cells). After a one month follow-up of the first four patients treated with MSC-NTF cells, no treatment-related adverse events were observed, indicating a short term safety of the treatment. Conclusion: Preliminary results of our ALS trial with autologous MSC-NTF cells after ex-vivo differentiation indicate that the used treatment protocols appear to be safe. Further analysis of the data during the following months of the trial, is needed to confirm the safety and efficacy of the proposed therapy. Trial registration: ClinicalTrials.gov (ClinicalTrials.gov Identifier: NCT01051882). The study is sponsored by Brainstorm Cell Therapeutics Ltd.

Poster Board Number: T-3315

DOPAMINERGIC NEURONS DERIVED FROM HUMAN DENTAL PULP STEM CELLS

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Aim / Background: Parkinson's disease, one of the serious neurodegenerative diseases, is caused by the loss of dopaminergic neuron. In order to overcome Parkinson's disease, stem cell therapy was suggested as an alternative treatment. Human dental pulp stem cells (hDPSCs) are one of the adult multipotent stem cells, which can differentiate into three germ layers including neurons. As hDPSCs can obtain noninvasive way by deciduous tooth, they could be a suitable autologous stem cell source. We identified that hDPSCs could differentiate into dopaminergic neurons under appropriate in vitro conditions. Methods: hDPSCs were cultured with dopaminergic neuron differentiation media and the dopaminergic neuronal characters were identified at each stage. With morphological changes, stem cell properties and potential to differentiation into dopaminergic neuron of hDPSCs were verified with immunocytochemistry (ICC), flow cytometry, Real time PCR and

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ELISA. Results: Differentiation of hDPSCs into dopaminergic neuron was verified according to the 5 stages. hDPSCs were proliferated at stage 1 and neurospheres were formed at stage 2. Subsequently at stage 3 and 4, NESTIN-positive cells were selected and expanded. Lastly at stage 5, cells were induced into dopaminergic neuron. On the protein level, the expression of stem cell maker (SSEA4) was observed at early stages and gradually decreased. The expression of early neural stem cell marker (NESTIN) and early neuronal marker (TUJ1) were maintained through the stages. The late neuron markers (GFAP and MBP) were highly expressed from stage 3. Most importantly, the dopaminergic neuron marker (Tyrosine Hydroxylase, TH) expression was found at stage 5. Flow cytometry results showed that expression of neuron-related markers was increased during the stages, while expression of SSEA4 was decreased. On the gene expression level, the expressions of SSEA4 (stem cell maker) and VIMENTIN (neural stem cell marker) were constant, while, the expressions of OCT4, NANOG (stem cell makers), NESTIN, PAX6 (neural stem cell markers) were observed gradually increased by time course, and the expressions of early (TUJ1, MAP2) and late neuronal markers (GFAP, MBP, O4) were significantly decreased at stage 5. TH was highly expressed from stage 4 and matured at stage 5. The quantification of TH was carried with ELISA and the value was 0.289±0.063ng/ml at stage 5.
Conclusions: We identified that hDPSCs induce efficient neural differentiation, and the induced neurons contain TH-positive ones producing a significant amount of TH. Therefore, hDPSCs can be an alternative cell source of autologous cell therapy for Parkinson's disease.

Poster Board Number: T-3316

HUMAN AMNIOTIC FLUID STEM CELL-DERIVED MUSCLE PROGENITOR FOR URINARY INCONTINENCE

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Aim / Background: The most promising treatment for stress urinary incontinence (SUI) can be a cell therapy. Even though muscle progenitors or myoblasts are considering as ideal cell type for therapy (because easily fuse into host tissue and form postmitotic multinuclear myotubes), these cells require an invasive process to obtain. To avoid the painful biopsy, we suggest human amniotic fluid stem cells (hAFSCs) as an alternative cell source. We established the optimum in vitro protocol for differentiation into the muscle progenitor cells, and consequently the progenitors were transplanted into injured urethral sphincter to analysis their therapeutic effect and safety in vivo. Methods: To develop the efficient differentiation system in vitro, we examined a commercial medium, co-culture or conditioned medium (CM) system, and evaluated the molecular variations with real-time PCR and immunocytochemical analysis. The *in vivo* therapeutic effect of the hAFSC-derived muscle progenitors was evaluated with the urinary incontinence mice model. Results: After being treated with CM, hAFSCs effectively developed into muscle lineage cells compared to other systems. Real-time PCR and immunocytochemical analysis showed that the CM treated hAFSCs significantly expressed the myogenic specific markers (PAX7, MYF5, MYOD, DESMIN). The muscle progenitors transplanted into the animal model were integrated into the host urethral sphincter tissue and stimulated host cell differentiation. With urodynamic analysis, muscle progenitors injected group showed significant increase leak point pressure and closing pressure compared to hAFSCs injected group. The functional result was confirmed with histological and immunohistochemical analysis demonstrating formation of normal-appearing circular muscle mass regeneration and expression of larger number of target markers than the control. In immunogenicity analysis, the muscle progenitor injection groups had negative HLA-DR expression, a scant CD8 lymphocyte aggregation and no teratoma formation. Conclusions: We identified that hAFSCs can effectively differentiate into muscle progenitor cells in CM and the hAFSC-derived muscle progenitor cells can represent an accessible cell source for damaged urethral sphincter regeneration.

Poster Board Number: T-3317

MICRORNAS SECRETED ISCHEMIC HEART INHIBIT SURVIVAL OF TRANSPLANTED RAT MESENCHYMAL STEM CELLS

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Stem cell therapy for repair of myocardial injury has essential limitations due to the poor survival of stem cells after cell transplantation. After transplantation of mesenchymal stem cells (MSCs), cell survival is inhibited by various factors secreted from ischemic surroundings after myocardial infarction. Recently microvesicles (MVs) have been known to mediate cellular communication. The goals of this study are to identify effects of miRNA secreted from ischemic heart for MSC survival. We demonstrated that MVs were secreted by ischemic hearts using transmission electron microscopy and MVs containing various miRNAs were isolated from hypoxic cardiomyocyte by real-time PCR analysis. To determine the roles of miRNAs in MVs isolated from hypoxic cardiomyocytes for MSCs survival, we investigated the survival of hypoxic MSCs after transfection of miRNA mimic *in vitro*. In various miRNAs-treated MSCs, the survival rate was 2-folds lower and cells were arrested in G1/S checkpoint. In addition to, the ratio of Bcl-2/Bax decreased 2-fold but caspase-3 activation was increased. Annexin V/PI assay was consistent results with the cell survival. These results indicated that secreted MVs from ischemic heart inhibited survival of implanted MSCs and provide evidence that the elimination of miRNAs secreted from ischemic hearts might be a novel strategy for increasing successful stem cell therapy via improving the survival of engrafted MSCs.

Poster Board Number: T-3318

RAT MESENCHYMAL STEM CELLS PROTECT HYPOXIC CARDIOMYOCYTES VIA SECRETION OF MICRORNA-125B MICROVESICLES

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Microvesicles (MVs), an extracellular vesicle, are secreted from all cells with microRNAs (miRNAs), which act mediators for cell-to-cell

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communications. Because microRNAs in MVs regulate cell differentiation, proliferation and immune systems, MVs from stem cells may be mechanical mediators for regenerative therapy such as many cytokines secreted from MSCs. Paracrine effects of MVs remain less understood in cell therapy for ischemic heart. Here, we showed that miR-125b in MVs secreted from MSCs protects hypoxic cardiomyocytes by inhibition of apoptosis. MicroRNA-125b MVs secreted from MSCs were isolated and added into hypoxic cardiomyocytes. In MVs-treated hypoxic cardiomyocytes, the cell viability and anti-apoptotic Bcl-2 expression were increased and caspase-3 activity and pro-apoptotic Bax expression were decrease, compared to non-treated cardiomyocytes. In addition, Annexin V and PI staining was decreased. Furthermore, these results were consistence with transfection of miR-125b mimic in hypoxic cardiomyocytes. Our results demonstrated that microvesicles secreted from MSCs protect hypoxic cardiomyocytes, and may help develop better clinical strategies for myocardial damage.

Poster Board Number: T-3319

HUMAN STEM CELL THERAPY FOR THE TREATMENT OF RADIATION - INDUCED NORMAL TISSUE DAMAGE

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In Europe, per year, 1.5 million patients undergo external radiotherapy. The late adverse effect of radiotherapy concern 5 to 10% of them. Eradication of these manifestations is crucial. We are strongly implicated in the field of regeneration of healthy tissue after radiotherapy or radiological accident. In 2004, in collaboration with Saint-Antoine Hospital (Paris, France), our first success in cell therapy was the correction of deficient haematopoiesis in two patients. The intravenous injection of Mesenchymal Stem Cells (MSC) has restored bone marrow micro-environnement necessary to sustain haematopoiesis after total body irradiation. From 2006 until 2011, we have obtained foremost success in cell therapy of radiation induced burns. In collaboration with the Percy hospital (Clamart, France) we have evidenced for the first time, the efficiency of MSC therapy in the context of acute cutaneous and muscle damage following irradiation in five patients. In 2007, we have also demonstrated that MSC treatment is a promise approach for the medical management of gastrointestinal disorder after irradiation. Three patients were successfully treated for consequences of over exposure for pelvic radiotherapy in 2007 and one patient in 2011. Evaluation of stem cell therapy combining different source of adult stem cells is under investigation. Key message of your presentation in 30-40 words : Clinical trials for treatment of radiation - induced normal tissue damage after radiotherapy or radiological accident using Mesenchymal Stem Cells (MSC) to restored haematopoiesis, acute cutaneous skin and muscle damages or consequences of pelvic radiotherapy. Key words: stem cell therapy, radiotherapy, healthy tissue, gastrointestinal disorder.

Poster Board Number: T-3320

LET-7B MODULATES SURVIVAL OF HYPOXIC HUMAN MESENCHYMAL STEM CELLS VIA THE REGULATION OF PARP

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Although mesenchymal stem cells (MSCs) represent a suitable source for therapy of heart failure, the poor survival of MSCs limited the efficacy of cell therapy. Cleaved Poly (ADP-ribose) polymerase(cPARP)-1plays an important role in cellular injury such as ischemia-reperfusion of the heart. PARP-1, endogenous enzyme, repairsthe broken DNA strand in nucleus due to oxidative stress, chromatinremodeling or cell death. But the cleavage ofPARP by caspase-3 inhibits PARP's DNA-repairing abilities. MicroRNAs (miRNAs) are short non-coding RNA and key regulator in various biological functions including cell differentiation, remodeling, cell proliferation, and cell death by negatively regulating gene expression. In this study, we found that caspase-3 was a possible target of let-7b, inhibiting the cleavage of PARP. Overexpression of let-7b increased cell survival by the regulation of PARP activity in MSCs. In MSCs transfected with let-7b mimic in hypoxic condition, the apoptosis-related factors such as p-AKT, p-MEK, p-ERK, Bcl-2, Bax, cytochrome-c, cPARP and Annexin V/PI were well regulated. These results indicate that let-7b regulates expression of PARP for repairing DNA strand and for increasing cell survival in hypoxic MSCs by inhibiting caspase-3 activity.

Poster Board Number: T-3321

HUMAN ADULT ADIPOSE-DERIVED STROMAL VASCULAR FRACTION AND HIGH DENSITY PLATELET CONCENTRATES IN BIOCELLULAR REGENERATIVE MEDICINE

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Biocellular regenerative medicine values the heterogeneous, undifferentiated cell population with fat tissues. High numbers of undifferentiated cells, ease and safety of access, and the native 3D adipose matrix, favors fat as a stem/stromal cells source. In translating from lab to bedside, an appreciation of potential uses of the cellular, structural, chemical and paracrine interactions within elements of the stromal vascular fraction (SVF) has gained attention. Understanding the potentials of platelet growth factors and signal proteins have led to combining the cell source, native adipose biomatrix (scaffolding), and signaling elements for wound healing and regeneration *In vivo*. Paper studies the components of AD-SVF from a cellular, structural, chemical, and mechanical viewpoint. As a huge microvascular bed, fat provides a vast pool of undifferentiated cells with complex signaling mechanisms. Comparative studies remain challenging, as standardization of terms and protocols remains elusive. Paper brings an understanding of the between clinical experiences and the complex mechanisms in play within various microenvironments. The makeup of the AD-SVF is key to

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the potential uses in tissue maintenance and repair. Importance of regulatory feedback and the niche to cellular proliferation and differentiation is presented. The ability to transfer the intact niche (microenvironmental) components within lipoaspirate samples, offers real potential in the clinical setting. Advantages of non-manipulated tissues relative repair potentials and the ability to deliver these elements within a "same surgical procedure"are outlined. Research activities have sought to isolate, concentrate and expand the component cellular groups. We believe provision of the entire "smorgasbord" of undifferentiated, multipotent cells and their native scaffolds may be of greater value than any one component. Letting the niche dictate its needs may be more effective than isolation of some parts. Highly concentrated platelet-rich plasma addition offers a diverse bioactive stimulus, providing an array of growth factors, signal proteins, cytokine/chemokines and lipokines. This is believed to enhance healing and regenerative capabilities acting primarily in a paracrine function. A true biocellular mixture can be safely and effectively delivered with the living bioscaffolding of the adipose tissue complex. It appears that available perivascular and ECM elements (AD-SVF), the heterogeneous, and tissue specific conditions offer a major importance for total organism function, homeostasis, and regeneration. Tissue repair, homeostasis, and regenerative capabilities represent an inseparable interaction resulting in integral functions of the microenvironment it supports. Understanding the multiple factors involved in human homeostasis and self-repair is rapidly providing information which will alter many existing paradigms in the practice of medicine and surgery in the coming years. Permitting the niche to dictate the cellular fates and use of many variable inflammatory elements is providing a safe and effective clinical option in many musculoskeletal and reconstructive applications.

Poster Board Number: T-3322

SUCCESSFUL ISOLATION AND EXPANSION OF HUMAN SYNOVIUM-DERIVED MESENCHYMAL STEM CELLS (MSCS) GREW OUT FROM TISSUE EXPLANT BY USING CHEMICALLY DEFINED SERUM FREE MEDIA STK1 AND STK2

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Synovium-derived mesenchymal stem cells (MSCs) provide a superior cell source for cartilage regeneration because of their capacity for self-renewal and chondrogenic potential. For effective cell-based therapy, it is important consistently to obtain sufficient numbers of transplantable cells with minimal safety concerns. Traditionally, MSCs were isolated from synovium tissue by using collagenase and/or other proteases and expanded in media containing fetal bovine serum (FBS) or autologous serum. However, enzymatic digestion is time consuming, especially when applied to large volumes of tissues, and it decreases cell viability due to proteolytic activity. In addition, the sensitivity of tissues to collagenase shows large inter-individual and sample-to-sample variations. On the other hand, there are potential risks associated with the use of FBS in clinical settings. Thus, the increasing number of clinical protocols for cell-based therapy underscores the need for non-enzyme treatment and serum-free media. In the present study, we developed new methods for isolation of human synovium-derived MSCs from tissues, and isolated MSCs were expanded in a chemically defined serum-free medium STK1 and STK2 (DS Pharma Biomedical Co., Ltd., Japan). STK1 was used in primary cultures; meanwhile STK2 was used after the 1st passage. We found that synovium tissue fragments could adhere onto plastic culture surfaces within a very short time and that fibroblast-like cells actively migrated from the explants. Morphologic analysis and surface markers expression of these cells (positive for CD44, CD73, CD90, CD105, CD166, negative for CD34, CD45, HLA-ABC) indicated their mesenchymal origin. After *in vitro* expansions, these cells were successfully induced into adipogenic, osteogenic, and chondrogenic lineages. The growth rate and colony-forming efficiency of explants-derived cells were higher than those of cells obtained by collagenase treatment. Furthermore, after primary culture, explant-derived cells yielded larger numbers of cells than those obtained by the conventional digestion method. At the 5th passage and 4-6weeks after seeding, total cell number increased >1,000,000,000-fold in serum-free cultures of explant-derived cells with STK1 and STK2. In conclusion, we obtained multipotent MSCs from the synovium tissue by combination of primary explant cultures and chemically defined serum-free media STK1 and STK2. This method is simple, time saving, reliable and gives a high yield of cells compared to the conventional methods, and can be used in stem cell therapy.

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EFFECTS AND SAFETY OF HUMAN ADIPOSE TISSUE DERIVED STEM CELLS ON OSTEOARTHRITIC CARTILAGE EXPLANTS *IN VITRO*.

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Introduction. Osteoarthritis (OA) is a chronic joint disease characterized by progressive destruction of articular cartilage and degeneration of the extracellular matrix. Matrix enzymes, such as metalloproteinases (MMPs) play a crucial role in the modulations of degradation and cell-matrix interactions. MMPs are controlled through activation of proenzymes and the inhibition of active enzymes by tissue inhibitors of metalloproteinases (TIMPs). Many studies imply the therapeutic potential of mesenchymal stem cells (MSCs) for cartilage repair. Adipose tissue derived stem cells (ADSCs) are close to MSCs from bone marrow in their characteristics, moreover, ADSCs are much easier available. Therefore, the development of ADSC therapy in OA, using their paracrine and chondroprotective effect would be advantageous. The present study is a part of the international project designed for elaboration of clinical procedure for the therapeutic application of ADSCs in OA. Materials and methods. To evaluate effects of human ADSCs for OA cartilage we performed *in vitro* ADSC cocultures with articular cartilage explants (CE) for 3 and 7 days. To further reproduce the OA conditions co-cultures were also stimulated with IL-1β. ADSCs were isolated under GMP conditions, and all cultures were performed with incomplete chondrogenic medium supplemented with human platelet growth factor-enriched plasma. Secretion of TIMPs, MMPs, and other factors was analyzed in coculture supernatants by ELISA. CE were examined by histochemical and immunohistochemical methods. Expression of genes associated with chondrogenesis was analyzed in CE by RT-qPCR. Results. Factors, including fibronectin, hyaluronic acid, TIMP-1, TIMP-2 and TIMP-3 as well as MMP-2, which are known for their beneficial effect on OA cartilage, were produced by CE and particularly by ADSCs on day 3 and

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to the higher level on day 7. On the contrary to the other MMPs, the suppressive role of MMP-2 in arthritis has been reported. No production of VCAM-1, MMP-1, MMP-3, MMP-9 and MMP-13 which are harmful in arthritis was determined in supernatants of ADSCs. Co-cultures resulted in changes of analyzed gene expression profile in CE, with the most pronounced up-regulation of COL1A1 gene, as compared to CE alone. Addition of IL-1β to co-cultures differentially modulated production of MMPs and TIMPs, as well as gene expression pattern, however, effects of ADSC on CE essentially remains similar. Histological and immunohistochemical examination have shown a tendency to extracellular matrix improvement in the osteoarthritic CE cocultured with ADSC, suggesting their chondroprotective effects of those cells. Conclusions. Secretory profile and results of histological analysis suggest safety and possible beneficial effects of ADSC in prevention of cartilage from degradation during OA. The up-regulation of COL1A1 gene in the presence of ADSCs implies stimulated initial stage of OA cartilage healing. Presence of IL-1β may modify interaction between ADSC and CE in a co-culture system.

Poster Board Number: T-3324

MESENCHYMAL STEM CELLS: APPLICATION FOR ENHANCING THE ENGRAFTMENT OF HUMAN HEMATOPOIETIC STEM CELLS

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To overcome the limitations of allogeneic hematopoietic stem cell transplantation (HSCT), such as graft rejection and graft versus host disease (GvHD), we conducted a study to identify a strategy for enhancing HSC engraftment during HSCT. Cotransplantation experiments with mesenchymal stem cells (MSCs) derived from adult human tissues, including bone marrow (BM), adipose tissues (AT), and umbilical cord blood (CB) were conducted. AT-MSCs and CB-MSCs suppressed T-cell proliferation in mixed lymphocyte preparations as effectively as BM-MSCs, implying that MSCs have immunosuppressive effects as one of common characteristics of MSC. We showed that AT-MSCs and CB-MSCs enhanced the engraftment of HSCs as effectively as BM-MSCs in NOD/SCID mice, suggesting that AT-MSCs and CB-MSCs can be used as alternative stem cell sources for enhancing the engraftment and homing of HSCs. CB-MSCs derived from different donors showed different degrees of efficacy in enhancing the engraftment of HSCs. To investigate the critical factors of MSCs used for enhancing the HSC engraftment, we identified the difference in gene expressions according to MSCs' heterogeneity and culture conditions using microarray analysis and real-time PCR in multiple donors of CB-MSC day 7 after plating at different cell densities. Culture conditions had greater effect than MSC' heterogeneity on the difference in the gene expression of the cells, though the difference in the gene expressions of CB-MSCs was identified among different donors. Especially, CXCR7 showed the significant difference in the gene expression according to culture conditions. When CXCR7 in MSCs plating at high cell density that increased the efficiency of the HSC engraftment more than MSCs plating at low cell density was inhibited, the HSC engraftment was not increased as much as MSC plating at high cell density. This result suggests that CXCR7, known as a chemokine receptor able to bind the chemokines CXCL12/SDF-1 and CXCL11, in MSCs could be the critical factor of MSCs for enhancing the HSC engraftment by MSCs In conclusion, our results showed that BM-MSCs, AT-MSCs and CB-MSCs preferentially enhanced the engraftment of HSCs. Thus, AT-MSCs and CB-MSCs could be an alternative source of stem cells for cotransplantation with HSCs in HSCT. Although each of the MSC populations exerted different influences on the engraftment of HSCs and the gene expression profiles, the difference in the gene expression of MSCs and the effect of MSCs for enhancing the HSC engraftment were predominantly observed in the culture conditions, implying that *ex vivo* expansion according to culture conditions can overcome MSC' heterogeneity. Especially, the effect of MSCs plating at high density that enhanced the HSC engraftment may come from CXCR7. These results represent an important step toward preparing appropriate MSCs used for emerging as a therapeutic strategy and understanding their cellular signal mechanisms of the HSC engraftments.

Poster Board Number: T-3325

FACTORS RELEASED BY HUMAN UMBILICAL CORD BLOOD-MESENCHYMAL STEM CELLS ENHANCE SURVIVAL OF HL-1 CARDIOMYOCYTES UNDER SIMULATED ISCHEMIA

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OBJECTIVES: Cardiac stem cell therapy may contribute to functional improvement of the infarcted heart, even in the absence of *de novo* formation of contractile cells. Saving ischemically damaged cardiomyocytes from cell death by paracrine survival stimuli is one suggested mechanism. Because such paracrine effects are difficult to quantify in vivo, we sought to investigate the protective stem cell activity in a conditioned medium - cell culture ischemia model. METHODS: Human umbilical cord blood-mesenchymal stem cells (CB-MSC) were expanded and conditioned culture medium was prepared. Cultivated murine HL-1 cardiomyocytes were subjected to combined oxygen glucose serum deprivation in CB-MSC conditioned medium or control medium. After 5 hours treatment the total remaining cells as well as apoptotic cells converting the poly-caspase substrate SR-VAD-FMK were quantified using an automated high content imaging system. In addition, morphological changes of the nuclei connected to apoptosis were assessed. Via MTS assay metabolic activity was determined. Activation of kinases Akt and Erk1/2 and transcription factor Stat3 was investigated by western blot after 3 and 5 hours treatment. RESULTS: After 5 hours oxygen glucose serum deprivation the rate of detached dead cells was significantly lower in CB-MSC conditioned medium compared to control medium (29±3% vs. 39±7%). Furthermore, the fraction of non-apoptotic cells was increased in CB-MSC conditioned medium. Cell viability assessed via MTS assay was enhanced significantly by 23±7% relative to control. CB-MSC conditioned medium further lowered nuclear shrinking and fragmentation, the major nuclear morphological changes associated with apoptosis. Phosphorylation of cell survival promoting kinases Akt and Erk1/2 was elevated when cells were kept in CB-MSC conditioned medium during oxygen glucose serum deprivation. Stat3 phosphorylation was enhanced significantly by factor 9±2 relative to control after 3 hours and declined to factor 6±3 after 5 hours. CONCLUSIONS: Factors released by human CB-MSCs protect cardiomyocyte-like HL-1 cells from the deleterious impact of oxygen glucose serum deprivation. This effect is associated with the activation of cell survival promoting kinases Akt and Erk1/2 and transcription factor Stat3. Studying the interaction between stem cells and ischemically damaged cardiomyocytes on a molecular level is indispensable to explain the beneficial effects of cardiac stem cell therapy. Our findings reinforce the thesis that paracrine mediated survival stimuli contribute to these effects.

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THE SAFETY AND EFFICACY OF HUMAN BONE MARROW-DERIVED CLONAL MESENCHYMAL STEM CELLS IN A RAT SPINAL CORD INJURY MODEL

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Mesenchymal stem cells (MSCs) are multipotent and differentiate into a range of cell types and are being tested for their regenerative potential. However, most preclinical and clinical studies have used a mixed population of mononuclear cells, which has led to confused results in the outcomes of clinical trials. In this study, we aimed to evaluate the safety and efficacy of human bone marrow-derived clonal mesenchymal stem cells (hcMSCs), homogeneously isolated by using subfractionation culturing methods (SCM), in a rat spinal cord injury (SCI). Human bone marrow-derived primary (hpMSCs) and clonal mesenchymal stem cells (hcMSCs) were labeled with PKH26 before freezing, and then kept in a nitrogen tank. The rat SCI model was made with using a vascular clip at T9 level. One hour after injury, approximately 1.5×10^5 cells were gently washed three times with PBS then transplanted into the lesion site. Rats were divided into four groups of sham, SCI+PBS, SCI+hpMSCs and SCI+hcMSCs. Motor function test was performed during 4 weeks post injury by BBB score. Rats were sacrificed at 1 day, 1 week, 2 weeks and 4 weeks and spinal cords were removed for histological analysis. Our results showed that the transplantation of hpMSCs and hcMSCs had no significant adverse effect on inflammatory reaction, compared with that of the SCI+PBS groups. In addition, transplantation of hcMSCs improved the recovery of hind limb locomotor function and the expression level of neurofilament protein, and reduced the cavity size.

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DESIGNING A CLINICAL PROGRAM FOR AUTOLOGOUS ADIPOSE DERIVED CELL THERAPIES IN CARDIAC PATIENTS

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Developing a cell therapy product for commercial use is a novel and complicated process. Determining the appropriate infrastructure for each stage of product development and testing can be crucial to taking the product to the clinic. Establishing appropriate timelines and meeting budgets demands can be a challenge for start-up companies with limited funding. This session will discuss the Bioheart experience in taking cell therapy products to the clinic and the challenges to ensure patient safety and financial security in the path the commercialization. Bioheart has collaborated with many groups to bring these therapies to patients including the Ageless Regenerative Institute and Regenerative Medicine Institute. In a recent protocol focused on congestive heart failure, more than 10 patients have been successfully treated using adipose derived stem cells. The cells are delivered directly into the heart muscle using a catheter. These patients have demonstrated on average, an absolute improvement of 13 percentage points in ejection fraction and an increase of 100 meters in their 6 minute walk distance at their 6 month follow up. This means that the patient's heart appears to be functioning better on echocardiogram. In addition, the patients have improved their exercise capacity which allows for a more active and normal lifestyle. This data is in agreement with the many years of pre-clinical animal studies that were completed by ARI and Bioheart demonstrating the safety and efficacy of this therapy including a study led by Keith March, MD, PhD, Director of the Vascular and Cardiac Center for Adult Stem Cell Therapy at the Indiana University. The adipose cells showed a tendency toward cardiomyocyte regeneration, prominent angiogenesis (growth of new blood vessels) and reduction in the infarction size. The prominent angiogenesis demonstrated by ADSCs, make them the perfect therapy for ischemic tissue.

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CLICAL OBSERVATION OF 8 CASES OF PATIENTS WITH JUVENILE RHEUMITOID ARTHRITIS TREATED BY AFFINITY UMBILICAL CORD MESENCHYMAL STEM CELLS

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Background: Juvenile rheumatoid arthritis(JRA) is a common rheumatic disease in childhood, which mainly characterizes chronic arthritis and relates all the body systems, also can cause the first factor for childhood mutilation and loss of sight. This disease can be divided whole boy type, multi-joint type and hyp-joint type, showing long time fever, rash, arthralgia, leukocytosis. The disease commonly involves childhood of 2-16 years, affecting their growth and development, and is very difficult to be cured. Objective: To observe the effect of using umbilical cord mesenchymal stem cell (U-MSC) to treat JRA and try to explore a new way for the disease treatment. Method: From March to November 2011, we collected 8 cases of JRA (according to the diagnostic standard of USA rheumatism society) patients to our hospital, 5 males, 3 females , ages from 2 to 12 years, their courses of disease had been for 1 to 3 years. The patients had been repeatedly instructed to use hormone, NSAIDS, DMAIDS and other drugs for therapy without good effect. We tested the patients' immune index containing T cell subgroup, Treg, and phlegmonosis index(IL-6,INF-Y,TNF-α,IL-17,TGF-β). We used U-MSC(supplied by Tianjin Heze Biological Technology Limited Company) 2×10^7/40ml,with hexadecadrol 2 mg intravenous infused to the patients. Before infusion we applied tanshinon, Luguapolypeptide, and bone growth factor to the patients for 1 week, and individually gave NSAIDS, DMALugua IDS(MTX, LEF,HCQ) and low dose of prednisone 5-10mg/d to the patients for therapy. Result: After using U-MSC for treatment 1 week to 4 months we followed up the patients, we found that 8 patients had no bad reaction and their blood routine, liver function, kidney function were all normal, and their fever rash, arthraligia, and body state were all improved. And we also could reasonably decrease their medicine use. The laboratory index showed 5 of the 8 patients' Treg up-regulation, and the inflammatory factor showed 4 of the 8 patients' IL-6, TNF-α, IL-17 level descended after treatment for 1 week, but after 4 months the IL-17 level raised gradually up. While other 4 of the 8 patients' IL-6,TNF-α,IL-17 level still decreased. Conclusion: U-MSC used to treat JAR could be a safe and prospective way , which may regulate immune disorder and make Treg up, make the inflammation factor down.

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Poster Board Number: T-3329

THERAPEUTIC APPLICATION OF HUMAN ADIPOSE STEM CELLS TO REPAIR CARDIAC DAMAGE AND DISEASE

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Stem cell therapy offers the potential for enhanced restoration of the loss of heart function associated with cardiac disease and infarcts that affects millions of people each year. Adipose stem cells (ASC) in particular represent a bountiful and readily accessible source of autologous adult stem cells with demonstrated capacity for cardiac lineage differentiation. We have previously demonstrated that the hematopoietic stem cell (CD34+LinNeg) sub-population of human whole bone marrow is crucial for the regenerative effects following injection of bone-marrow derived samples into infarcted hearts in a murine model of cardiac ischemia. We now wish to assess whether re-programming stem cells towards the cardiogenic lineage prior to use in therapy could potentially further enhance tissue repair and the restoration of cardiac function. Since ASC are multipotent and more abundant and accessible than HSC, we have elected to use adipose-derived samples in the present study. Like bone marrow derived samples enriched in HSC, adipose derived samples enriched in ASC (CD90+CD105+CD45-CD31-) were observed to effect improvement in cardiac output (LVEF) and reduce scarring (mean percentage area fibrosis of total heart) following inter-cardiac transplantation into SCID mice directly after induced myocardial infarction. To assess whether re-directing ASC towards the cardiac lineage prior to transplantation may increase the efficacy of stem cell-based therapies to treat cardiac damage, we constructed a lentiviral vector engineered to constitutively express the re-programming factors Gata4, Mef2c, and Tbx5. We are able to efficiently transfect human ASC samples with this vector, and detect up-regulation of cardiogenic lineage markers 14 days post-transfection. These results lay the groundwork to test the relative capacity of ASC and re-programmed ASC to facilitate repair from myocardial infarcts.

Poster Board Number: T-3330

ENGINEERING OF A HUMAN DEATH-INDUCIBLE MESENCHYMAL STEM CELL LINE; TOWARDS STANDARDIZED AND SAFE CELLULAR THERAPY.

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Introduction
Human bone marrow-derived Mesenchymal Stem/Stromal Cells (hMSCs) are promising candidates in regenerative medicine, potentially indicated for tissue repair, tumour targeting, and treatment of immune-related diseases. However, the high inter-donor variability, combined with the intrinsic heterogeneity of hMSCs preparations and the lack of quality standards, limits a coherent exploitation of their therapeutic potential. If the generation of immortalized hMSCs could solve cell-source based problems, it also raises safety concerns regarding their stability / tumorigenicity. Towards the standardized and safe clinical use of hMSCs, the aim of this study is thus to generate hTERT-immortalized hMSCs including an inducible suicide system. The engineered cell line should conserve the properties of primary hMSCs, while being efficiently inducible toward apoptosis in vitro and *in vivo*. Materials & Methods Primary hMSCs were transduced at early passage with a lentivirus carrying hTERT. A retrovirus-mediated transduction allowed the integration of an inducible, modified apoptotic caspase 9 gene into the immortalized population. A clone having stably integrated the death-device was isolated and expanded. Cells were analyzed phenotypically, and characterized for their differentiation capacity (adipogenic, osteogenic, chondrogenic), tumorigenicity (RT-PCR analysis and *in vivo* study), and their immunogenicity. The killing efficiency of the death-device was assessed in vitro by Annexin-V/PI staining and *in vivo* by using a luciferase reporter system. *In vivo* studies were based on ectopic implantation in nude mice. Additional viral transductions (i.e., for VEGFα, BMP-2) were performed to validate the feasibility to introduce further functionalities in the engineered cell line. The stability of the transgenes was assessed at different passages and after each transformation. Results Fresh hMSCs were successfully immortalized (>280 doublings over more than 1 year) and stably transformed with an inducible-death device. The induction of the death system led to an efficient killing in vitro (>95%) and *in vivo*. Despite an extensive expansion and even following additional transductions, the generated clonal cell line was phenotypically identical to primary hMSCs. Cells could also differentiate toward osteoblastic, adipogenic and chondrogenic lineages with similar or increased efficiency when compared to their primary counterparts. Engineered hMSCs displayed no immunogenic properties (no allogenic CD8+ activation) and no tumorigenicity in vitro (normal RNA level of p53, p21, cMyc and Rb1 genes) and *in vivo* (absence of tumor development 3 months post implantation). Conclusion We engineered a stable hMSCs line maintaining typical functionalities and inducible into apoptosis upon demand. The cell line represents a standardized cell source for a variety of possible clinical indications with increased safety margins. In fact, cells can be extensively tested and screened prior to clinical use and potentially killed once they have elicited their function *in vivo*. The study opens the general perspective of using genetic tools not only to modify cellular function, but also to standardize and increase the safety of cellular therapy.

Poster Board Number: T-3331

AGING CAUSES ACTIVATION OF UROKINASE SYSTEM AND MMPS IN HUMAN ADIPOSE-DERIVED MESENCHYMAL STEM CELLS

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Introduction. Mesenchymal stem cells, including adipose-derived mesenchymal stem cells (ADSCs) actively participate in tissue repair and regeneration due to their differentiation capacities as well as abilities to stimulate the growth of blood vessels and nerves. ADSCs produce a wide range of growth factors, which stimulate migration and surviving of endothelial cells and axons. These cells also produce matrix proteases, including metalloproteinases (MMPs) and urokinase, which mediate extracellular matrix remodeling and proteolytic activation of growth factors. Aging is associated with impaired tissue repair, increased extracellular matrix to cells ratio as well as changes in the composition of extracellular matrix. We previously demonstrated that ability of ADSCs to stimulate blood vessel growth decreases with age due to deteriorated secretion of angiogenic growth factors. Age-associated changes in proteases secretion by ADSCs was not known, therefore the aim of our study was to access how aging affects urokinase system and MMPs production by human ADSCs. Methods. ADSCs were isolated

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from subcutaneous fat tissue samples collected during surgical procedures from 61 patients (aged 2.5 to 82 years old, mean age 56.6±15.6 years). Cells were characterized as MSCs according to their immunophenotype and differentiation capacity. Telomere length as well as urokinase (uPA), its receptor (uPAR), plasminogen activator inhibitor-1 (PAI-1), MMP2, MMP9 expression in cultured ADSC were assessed using real-time PCR. uPAR surface expression was estimated by flow cytometry. Content of pro- and active forms of MMP2 and MMP9 were determined by zymography. Level of PAI-1 secreted by ADSCs was accessed in conditioned medium by ELISA. Results. Expectedly, ADSCs from aged patients had shorter telomeres (correlation between relative telomere length and age r = -0,56, p=0,01). Cells of elder patients had elevated uPAR and PAI-1 gene expression (correlation between expression level and age r=0,44, p=0,001 and r=0,46, p=0,01, correspondingly) as well as uPAR surface expression (r=0,65, p=0,01). These changes also inversely correlated with telomere length in ADSCs (r=-0,35, p=0,04 for uPAR mRNA and r=-0,54, p=0,01 for PAI-1 mRNA). We found that ADSC with shorter telomeres produced significantly more PAI-1 to conditioned medium (r=-0,41, p=0,02). Content of pro- forms of MMP2 and MMP9 was higher in ADSC obtained from patients older than 60 years compared to younger patients (p<0,05) and we observed similar tendency for active forms of MMP2 and MMP9, but it was not statistically significant. Conclusions. Our data suggest that aging is associated with up-regulation of urokinase system and MMPs in ADSC. Elevated proteolytic activity of ADSCs from elder patients can reflect cells adaptation to the changes in extracellular matrix during aging and can also act as a compensatory mechanism, providing more efficient activation of fewer amounts of secreted growth factors.

Poster Board Number: T-3332

HUMAN NAÏVE UMBILICAL CORD BLOOD-DERIVED MESENCHYMAL STEM CELLS ARE EFFECTIVE FOR TREATMENT AS WELL AS PREVENTION OF GVHD IN NSG MOUSE MODEL

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Recently, the studies about GVHD prevention and treatment using mesenchymal stem cells are proceeding but the therapeutic effects are still controversial. Therefore, this study was designed to address that human naïve umbilical cord blood-derived mesenchymal stem cells (UCB-MSC) has effect on treatment as well as prevention in xenogeneic GVHD model using NSG mouse. To investigate the prevention effect of UCB-MSC, 5×10^5 naïve UCB-MSC were transplanted via tail vein with single (day 0) or repeat dose (day 0, 3, 6). After 60 days, while the survival rate of UCB-MSC single injection group was 12.5%, UCB-MSC repeat injection improved the survival rate up to 62.5%. To observe the therapeutic effect of UCB-MSC, they were transplanted via tail vein with single (day 18) or repeat dose (day 18, 21, 24) after GVHD onset. Naïve UCB-MSC increased the survival rate up to 50% with single injection. After observing until 60 days, we analyzed plasma of all live mice to investigate immune suppressive molecules secreted from UCB-MSC. In this analysis, we could detect PGE2, TGF-β1 and IL-10 and their levels were significantly increased compared to control group that was not administered UCB-MSC. In addition, suppressor T cells increased 44.78% in repeat injection of preventing group and 40.75% in single injection of treating group. Taken together, our data confirmed that human naïve UCB-MSC are effective at treating as well as preventing GVHD by secreting immune suppressive molecules and increasing suppressor T cells. This study as a clinically applicable model suggests that repeat injection is needed for GVHD prevention but the therapeutic effect is enough with single injection.

Poster Board Number: T-3333

SOLUBLE CCL5 FROM BM-MSCS IN THE BRAINS OF AD MICE WITH AB DEPOSITION

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Microglia have the ability to eliminate amyloid β (Aβ) by a cell-specific phagocytic mechanism, and bone marrow (BM) stem cells have shown a beneficial effect through endogenous microglia activation in the brains of AD mice. However, the mechanisms underlying BM-induced activation of microglia have not been resolved. We show that BM-derived mesenchymal stem cells (MSCs) induced the migration of microglia when exposed to Aβ *in vitro*. Cytokine array analysis of the BM-MSC media obtained after stimulation by Aβ further revealed elevated release of the chemoattractive factor, CCL5. The CCL5 was increased when BM-MSCs were transplanted into the brains of Aβ-deposited AD mice, but not normal mice. Interestingly, alternative activation of microglia was associated with elevated CCL5 expression. Furthermore, by generating a chimeric mouse, we ascertained that the activated microglia resulted from endogenous BM cells that were recruited into the brain by CCL5. Additionally, we observed that neprilysin (NEP) and IL-4 derived from the alternative microglia were associated with a reduction in Aβ deposition and memory impairment in AD mice. These results suggest that the recruitment of the alternative microglia into the brain is driven by CCL5 secretion from the transplanted BM-MSCs, which itself is induced by Aβ deposition in the AD brain. This work was supported by the grants for the Bio & Medical Technology Development Program (2010-0020234) and Basic Science Research Program (2010-0003949, 2010-0009421) funded by the National Research Foundation (NRF) of the Ministry of Education, Science and Technology, Republic of Korea.

Poster Board Number: T-3334

PROSPECTS OF USING MOUSE BONE MARROW MESENCHYMAL STEM CELLS OVEREXPRESSING VEGF IN A MURINE DOUBLE TRANSGENIC MODEL OF ALZHEIMER'S DISEASE

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Objectives: Our proposal is to investigate the effect of intracerebral transplantation of mouse bone marrow mesenchymal stem cells overexpressing VEGF (MSC-VEGF), an important angiogenic and neurotrophic factor, on the vasculature and neurodegeneration in double transgenic mice model of Alzheimer's disease (2xTg-AD) with APPswe/PS1dE9 mutation. Since the Aβ peptide inhibits angiogenesis and promotes the degeneration of blood capillaries, our hypothesis is that mesenchymal stem cells overexpressing VEGF (MSC-VEGF), when transplanted into the brain, might have a potential to increase the vascularization and angiogenesis rate in 2xTg-AD animals. Methods: Bone marrow mesenchymal stem cells from 6 weeks old C57BL6/EGFP mice were transfected with uP-

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VEGF vector, constructed with the promoter, enhancer and intron 1 of cytomegalovirus to express human VEGF165. Transfection was performed using electroporation and expanded at the 14th passage. Transgenic 2xTg-AD animals at 6, 9 and 12 months old were transplanted with MSC-VEGF and MSC. To the the control groups was administered saline solution. Forty days later, the animals were tested for Open Field, Social Recognition and Plus-maze Discriminative Avoidance behavioral tasks, to access locomotion, novelty exploration, learning and memory. At the end of the behavioral tests, the animals were perfused and their brains were analyzed for vascularization, neurogenesis, cortical atrophy, neuroprotection and clearance of Aβ, by immunohistochemistry and immunofluorescence for CD31, DCX, NeuN, Iba-1, GFAP and Aβ6E10, respectively. Results: Transplantation of MSC-VEGF promoted neovascularization in 2xTg-AD animals, possibly by a paracrine effect. MSC-VEGF treatment recovered the innate interest in the novelty exploration in 12 months old 2xTg-AD animals, improved social recognition memory in 6 months 2xTg-AD animals and sustained learning activity at 9 months of age. Both MSC and MSC-VEGF treatment were able to improve motor deficits and recover long-term memory in 9 and 12 months old transgenic animals. There was retention of long-term memory, independent of the genotype, age and treatment. Conclusions: The beneficial effect of MSC-VEGF transplantation is related to the paracrine action of factors, mainly VEGF, which favored the neovascularization and the proliferation, viability and migration of endothelial cells. Thus, this modulation ultimately promoted improvement in cognitive deficits of 2xTg-AD animals. In addition to contribute to the understanding of the participation of bone marrow stem cells in angiogenic mechanisms in the brain of 2xTg-AD transgenic animals, this study has important therapeutic implications on angiogenesis damage, present in neurodegeneration promoted by Alzheimer's disease.

Poster Board Number: T-3335

INTERLEUKIN-17 DRIVEN PD-L1 EXPRESSION ON MOUSE MESENCHYMAL STEM CELLS AMELIORATE EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS DEVELOPMENT

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Recent studies indicated that *in vivo* administration of mesenchymal stem cells (MSCs) could significantly ameliorate the pathogenesis of experimental autoimmune encephalomyelitis (EAE), however, the mechanism by which MSCs exert their therapeutic effect remains elusive. Since IFNγ,TNFα and IL17A are reported to be crucial in the development of EAE, we stimulated MSCs with different combinations of these three cytokines. We found that PD-L1, a B7 family molecule with the ability to inhibit the function of T cells, was induced upon IFNγ and TNFα stimulation. Interestingly, when IL-17A was added, PD-L1 expression was further increased by several folds. This synergistic induction of PD-L1 in MSCs was abolished by blocking or depleting inducible nitric oxide synthase (iNOS) which is known to play a critical role in MSC-mediated immunosuppression. Moreover, addition of nitric oxide (NO) donor into iNOS deficient MSCs could restore the synergistic effect. Although administration of MSCs could ameliorate EAE in mice, co-adminstration of antibodies to PD-L1 with MSCs was found to abolish the therapeutic effect of MSCs. The effect of anti-PD-L1 is exerted through MSCs since anti-PD-L1 alone showed no effect. The key role of PD-L1 in MSC-based EAE therapy was also confirmed by the observation that iNOS deficient MSCs are not as effective as wild type MSCs in EAE mice. Therefore, PD-L1 expression is indispensable for MSCs-based therapy of EAE.

Poster Board Number: T-3336

COMBINED EFFECTS OF HEMATOPOIETIC PROGENITOR CELL MOBILIZATION FROM BONE MARROW BY G-CSF AND AMD3100, AND CHEMOTAXIS INTO THE BRAIN USING SDF-1A IN AN ALZEHEIMER'S DISEASE MOUSE MODEL

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Transplantation of bone marrow-derived stem cells (BMSCs) has been suggested as a potential therapeutic approach to prevent neurodegenerative diseases, but it remains problematic due to issues of engraftment, potential toxicities, and other factors. An alternative strategy is pharmacological-induced recruitment of endogenous BMSCs into an injured site by systemic administration of growth factors or chemokines. Therefore, the aim of this study was to examine the effects of therapy involving granulocyte colony stimulating factor (G-CSF)/AMD3100 (CXCR4 antagonist) and stromal cell-derived factor-1α (SDF-1α) on endogenous BM-derived hematopoietic progenitor cell (BM-HPC) recruitment into the brain of an Alzheimer's disease (AD) mouse model. To mobilize BM-HPCs, G-CSF was injected intraperitoneally and boosted by AMD3100. Simultaneously, these mice received an intracerebral injection with SDF-1α to induce migration of mobilized BM-HPCs into brain. We found that the memory deficit in the AD mice was significantly improved by these treatments, but amyloid β deposition was unchanged. Interestingly, microglial activation was increased with alternative activation of microglia to a neuroprotective phenotype. Furthermore, by generating an amyloid precursor protein/presenilin 1-green fluorescent protein (GFP) chimeric mouse, we ascertained that the GFP positive microglia identified in the brain were BM-derived. Additionally, increased hippocampal neurogenesis and improved memory was observed in mice receiving combined G-SF/AMD3100 and SDF-1α, but not in controls or animals receiving each treatment alone. These results suggest that SDF-1α is an effective adjuvant in inducing migration into brain of the endogenous BM-HPCs, mobilized by G-CSF/AMD3100, and that the two can act synergistically to produce a therapeutic effect. This approach warrants further investigation as a potential therapeutic option for the treatment of AD patients in the future (*Stem Cells,* 2011, 29(7):1075-89, *Co-correspondence to Bae JS or Jin HK).

Poster Board Number: T-3337

COULD DONOR MULTIPOTENT MESENCHYMAL STROMAL CELLS DIRECTLY MODIFY THE NATURAL EVOLUTION OF RETINAL DAMAGE IN DIABETIC MICE?

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Diabetes mellitus is a complex disease that has become an epidemic at worldwide level. Major medical advances have substantially improved the management of the disease, extending patient's survival. However, this also carries an increased risk to develop chronic macro- and microvascular complications. Among them, diabetic retinopathy (DR) is the most frequent and has remained the leading cause of visual loss by the past two decades. Local

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inflammation and reactive species production play a pivotal role in the pathogenesis of DR. These noxas are associated with the loss of pericytes and neuronal cells in the early stage of DR and may also be the driving force in the continued insult to the retina during the latter stage of the disease. Nowadays, there is no medical intervention that could prevent or delay the onset of DR. All therapeutic options involve highly invasive techniques and are applicable only in advanced stages of the disease. Therefore, the generation of less invasive and more effective strategies to prevent or delay the onset of DR is needed. Stem cell-based therapy represents an emerging therapeutic approach by which vascular and neuronal disease may be treated. Among that, multipotent mesenchymal stromal cells (MSCs) are highly attractive since they have been used to regenerate damage tissues and treat inflammation related diseases. Taking into account the mechanisms that trigger the initial steps of DR, the administration of MSCs could have favorable effects because they might (i) differentiate into pericytes and neuronal cells, replacing the dead ones; (ii) be neuro and vascular protective by the secretion of trophic factors; (iii) reduce oxidative damage by the scavenge of reactive oxygen species; and (iv) reduce inflammation by the secretion of anti-inflammatory cytokines. Therefore, the aim of this work was to evaluate the direct contribution of systemically administered MSCs in the natural evolution of DR. For this, mice with type 1 diabetes (T1D), in which we have previously proved that the administration of MSCs have no impact in blood glucose level, received 0.5x106 MSCs at a time in which all the pro-damage mechanisms are present but retinal histology is still preserve. First, we studied -by flow cytometry- the biodistribution of donor MSCs in mice with T1D. For this, donor cells were isolated from isogenic mice that express GFP (MSCsGFP). Three months post-administration, MSCsGFP were mainly found in the eyes of T1D mice, and in minor degree in other organs severely damaged during the disease like heart, kidney and pancreas. Second, we analyzed -by histology- the presence of structural changes in the retina. Whereas untreated T1DM mice exhibit structural alterations (including thickening in the IPL, INL and ONL retinal layers, and reduction in cell nuclei in the GCL retinal layer) in MSC-treated T1D mice retinas were architecturally organized as in normal mice. Finally, we assessed -by qRT-PCR- the level of molecular marker characteristic of DR including (i) markers of thickening of the vascular basement membrane; (ii) markers of inflammation; and (iii) markers of angiogenesis. Irrespective if T1D mice received or not MSCs, these markers were overexpressed in the retina in comparison with normal mice. Overall, our results show that in T1D mice systemically administered MSCs mainly home into the eyes and prevent the structural damage of the retina. Additional experiments are in course to clarify the mechanisms associated to the observed therapeutic effect.

Poster Board Number: T-3338

ADIPOSE-DERIVED STEM CELLS TRANSPLANTATION WITH LOSARTAN TREATMENT ENHANCE MUSCLE REGENERATION IN INJURED MDX MICE

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Duchenne muscular dystrophy (DMD) is a recessive X-linked form of muscular dystrophy, which results in muscle degeneration, difficulty walking, breathing, and death. Muscle-derived stem cells (MDSCs), myoblasts, hematopoietic stem cells, adipose-derived stem cells (ASCs), and induced pluripotent stem cells (iPSCs) have been used as a source for stem cell therapy in the treatment of DMD. Specially, ASCs as a material for stem cell therapy have a variety of advantages. For successful ASC transplantation, it is very important to make a good stem cell niche. Losartan is one of the drugs that may improve the stem cell niche, by attenuating the transforming growth factor-β (TGF-β) signaling pathway. The ASC transplantation with losartan treatment can be an effective method to treat DMD. Therefore, the aim of this study was to demonstrate whether the ASCs transplantation with losartan treatment in injured-mdx mice improves muscle regeneration. Gastrocnemius and soleus muscles of mdx mice were lacerated, and then, mouse ASCs were locally transplanted to the injured muscles of the mdx mice that were treated with or without oral losartan. ASCs transplanted into injured muscles of mdx mice directly differentiated in myofibers and dystrophin expression in the ASCs was identified both *in vivo* and *in vitro*. Remarkable muscle regeneration and inhibition of muscle fibrosis were also observed in the ASCs and losartan combined treatment group. Consequently, the present study shows that the stem cell therapy using ASCs with losartan improved muscle regeneration in injured-mdx mice.

Poster Board Number: T-3339

AUTOLOGOUS DENTAL PULP STEM CELLS IN REGENERATION OF DEFECT EXPERIMENTALLY CREATED IN CANINE PERIODONTAL TISSUE

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Until now, the bone regenerative capacity of dental pulp stem cells (DPSCs) has been investigated, however no investigation has evaluated DPSCs in the regeneration of periodontium that consisted of cementum, periodontal ligament (PDL) and supporting bone. In this study, we investigated the effect of DPSCs on regeneration of periodontal tissue defect experimentally-created in canine model. Canine maxillary premolar were cut around the root-enamel boundary, the pulp tissues were gently collected and then subjected to enzymatic digestion. The digest was then plated and propagated until homogenous fibroblastic cells dominated the culture. In parallel surgically-created mesial three-walled periodontal defects with ligature-induced periodontitis were produced bilaterally in the first lower premolar teeth of ten mongrel canines. Simultaneously, DPSCs were derived from maxillary premolar teeth of the same animals. Four weeks after creation of the periodontitis model, on one side, autologous passaged-3 DPSCs combined with Bio-oss were implanted as the test group. On the other side, only Bio-oss was implanted as a control. Eight weeks after surgery, regeneration of the periodontal defects was evaluated both histologically and histomorphometrically in terms of bone,PDL and cement formation. The pulp stem cells appeared fibroblastic in morphology throughout the cultivation period. They possessed differentiation potential into bone, cartilage and adipose cell lineages. The majority of cells tended to express CD90 and CD44 surface

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antigens. CD146, SSEA-4 and anti-macrophage were expressed in very low percentages in the studied cells. The loading efficiency of DPSCS onto Bio Oss was about 60%. Histologically, in all test specimens (ten defects), regeneration of cementum, bone and PDL was observed. In the control groups although we observed the regeneration of bone in all defects, the formation of cementum was seen in nine defects and PDL was seen in eight defects. Histomorphometric analyses showed that the amount of regenerated cementum and PDL in the test groups (3.83±1.32 and 3.30±1.12 mm, respectively) was significantly higher than the control groups (2.42±1.40 and 1.77±1.27 mm, respectively; $P<0.05$). In conclusion, biocomplex consisting of DPSCs and Bio-oss would be promising in regeneration of periodontal tissues.

Poster Board Number: T-3340

INTRACAROTID INJECTION OF ADIPOSE TISSUE-DERIVED PLATELET-DERIVED GROWTH FACTOR RECEPTOR B-POSITIVE CELLS IN CANINE MODEL: FEASIBILITY OF CELL DELIVERY UNDER MANNITOL-INDUCED BLOOD-BRAIN BARRIER OPENING

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PDGFRβ-positive pericytes is a multipotent mesodermal stem cell, which can be easily isolated from adipose tissue-derived stromal cells. The purpose of this study is to evaluate the safety and feasibility of intracarotid injection of adipose tissue-derived PDGFRβ-positive (AT-PDGFRβ$^+$) cells in canine model after mannitol-induced transient blood-brain barrier (BBB) opening. The canine subcutaneous adipose tissue for normal canine was prepared after the step of acquisition, magnetic sorting, transfection by lenti-GFP and proliferation. The stable proliferation of AT-PDGFRβ$^+$ was achieved at the condition of DMEM / F12 + 5%FBS + N2 supplement without growth factors. Normal canines (25kg, n=6) underwent intracarotid injection of hyperosmolar mannitol (20%, 50cc, 1g/kg) followed by 5x10^6 autologous PDGFRβ$^+$ cells. In first protocol (n=2), cells were mixed with 2ml of normal saline and infused for 1 min followed by hand injection of cells. In second protocol (n=4), cell solution within normal saline 50 cc plus heparin solution 1,000 unit, were dripped slowly using pressure bag during 10 min. Serial angiogram showed no evidence of thromboembolic complication. At each step of the procedure, the monitored mean arterial pressure, heart rate, and O2 saturation did not change. On blood test, the leukocyte count, serum calcium, serum potassium and total protein level is decreased at immediate post-infusion as compared to baseline, but the other complete blood count, electrolyte, liver and renal batteries are within normal range including CRP. On neurological scales, all six dogs showed intact motor and consciousness. The brain MRI of 1 day and 7 days showed small sized infarct in two dogs. The incidence of small sized infarct was 50% (additional) in the protocol 1, and 25% (transient) in the protocol 2. In conclusion, the intracarotid infusion of AT-PDGFRβ$^+$ cells under preparation of hyperosmolar mannitol is a feasible and safe method for the cell delivery.

Poster Board Number: T-3341

CHARACTERIZATION AND SAFETY PROFILE OF CANINE ADIPOSE DERIVED STEM CELLS

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Recent scientific achievements in the cell and molecular biology have promoted development of autologous stem cell therapy offering comprehensive possibilities for the treatment of human and animal diseases and dysfunctions. This therapy, as every novel method, is related to the certain risks, for example, disturbance of liver functions, thrombosis, hemorrhage and edema. Hence, before the clinical use of stem cells, safety tests must be performed at first. This study characterizes the properties of canine adipose-derived stem cells (ADSCs), cultured in the presence of autologous serum, and their safety profile after injection into the dog's bloodstream. Adipose tissue from a healthy adult Beagle dog was used to isolate adipose-derived stem cells that were cultivated and successfully propagated in a medium supplemented with the dog's autologous serum. After the second passage cells were frozen and then thawed for characterization and future examination. Fraction of obtained ADSCs was cultivated under hypoxic conditions (ADSCsA) and characterized in contrast to cells cultured at atmospheric oxygen level (ADSCsB). Both types of cells were plastic adherent spindle-shaped cells with fibroblastoid morphology. Telomeres of ADSCsA were elongated by 12,4 % and expression of surface marker genes CD73, CD90, CD105, CD34 and pluripotency gene NANOG was higher than in ADSCsB. Immunomodulatory properties of ADSCs A and B were compared by blast transformation reaction using dog's peripheral blood mononuclear cells. Significant suppression of T lymphocyte proliferation was induced in a dose dependent manner by both types of cells, but ADSCsA showed more pronounced immunosuppressive effect than ADSCsB. To test the safety of ADSCs cultured under low oxygen, at first the cell culture freezing medium containing dimethyl sulfoxide (DMSO) was injected into the dog's bloodstream followed by the therapeutic dose of ADSCsA (1x10^6 live cells/kg) and the repeated injection of five times higher dose (5x10^6 live cells/kg) after a month. No significant side effects were observed after these experiments and practically all the tested physiological, biochemical and blood circulation parameters were within normal limits. Exceptions were elevated levels of bilirubin in the blood as well as bilirubin and urobilinogen in the urine, observed 24 hours and 3 days after the injections. These might have arisen from the effect of metabolic processes of DMSO on biliary excretion. Obtained results indicate that canine ADSCs are safe and can be further developed for therapeutic purposes.

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Poster Board Number: T-3342

LOW SERUM CULTURED ADIPOSE-DERIVED MESENCHYMAL STROMAL CELLS AMELIORATE RAT MODEL WITH ZYMOSAN INDUCED SEVERE PERITONITIS

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Background; Peritoneum dialysis (PD) is one important therapy for end-stage kidney disease (ESKD). However, patients cannot continue PD therapy for an extended period because of ultra filtration (UF) failure, infection, or other complications. One of the reasons for UF failure is submesothelial fibrosis after peritonitis. Above all, fungal peritonitis is a major problem as the fibrosis can progress rapidly and it may evolve into encapsulating peritoneal sclerosis (EPS) even after the peritoneal dialysis catheter has been removed. We have developed an original culture system for low serum cultured adipose-derived mesenchymal stromal cells (LASCs). We have also reported the therapeutic potential of LASCs, especially immunomoduratory effect, in various animal models. In this study, we used rat peritonitis model induced by zymosan administration after peritoneal scrape accompanied complement activation and attempted to treat the model by LASCs. Methods; The fungal peritonitis was induced in the rat model by administrating zymosan daily for 5 days after scraping the peritoneum mechanically. The rats were divided into two groups; LASC (L group) or vehicle (V group) administration intraperitoneally with PD solution(1.5% glucose, neutral liquid) every day. On day 5, rats were euthanized and the peritoneums were harvested, then the thickness of the peritoneum, the infiltration of inflammatory cells, and the deposition of the complement were compared between the groups. To trace LASCs injected into peritoneum cavity, CFSE labeled LASCs were administered to the zymosan model, and then assessed by immunofluorescent staining. For the *in vitro* study, primary cultured mesothelial cells (MCs) were co-cultured with LASCs directly or indirectly in trans-well, and we observed the interaction. Results; On day 5, microscopic findings in L group had less plaques and less edematous. Histologically, the thickness of the peritoneum, the infiltration of inflammatory cells and the deposition of complements (both C3 and membrane attack complex (MAC)) in L group was significantly more reduced than those in V group. In addition, the mesothelial cell layer on the peritoneal surface in L group recovered more quickly compared with that in V group. Also, the layer in L group showed complement regulatory factors (CD55, Crry, CD59) clearly. In the tracing study, LASCs were laid mainly on the surface of peritoneum along with the recovered MCs. For the *in vitro* study, MCs directly co-cultured with LASCs proliferated earlier than those cultured without LASCs. The effect was also observed when the MCs were separated from LASCs by the trans-well insert. Conclusions; Administration of LASCs into the peritoneal cavity suppressed the inflammation of peritonitis induced by zymosan, and the mesothelial cell layer in L group recovered earlier than that in V group. This data suggests that LASCs have the multiple effects on peritoneal damage. In the future, LASC therapy may be useful for treating peritoneal injury during fungal infection of the peritoneum.

Poster Board Number: T-3343

STEMNESS AND STEM CELL RELATED GENE EXPRESSION PROFILE OF THE WOUND ENVIRONMENT IN PATIENTS WITH CHRONIC WOUND

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The role of endogenous stem cells in tissue repair has been explored in clinical and pre-clinical studies. Mesenchymal stem cells (MSCs) enhance tissue repair by up regulation of genes that modulate excessive inflammation and by providing an environment which enhance the proliferation and differentiation of tissue-endogenous stem/progenitor cells. For efficient and targeted therapy of the chronic wound, its niche must be explored to identify key cellular and molecular factors involved in the process. However, it has not been known if chronic non-healing patients show different profile in the expression of stem cell markers, stem cell related genes, and signaling molecules compared to patients who progress to healing. In the present study, we assessed stemness markers and MSC specific and related gene expression profile in patients with chronic non-healing wound. During debridement, a routine wound care procedure of removal of tissue from in and around the wound to optimize wound healing, tissue samples was collected mRNA extracted, and a standard quantitative RT-PCR along with customized stem cell PCR arrays were conducted. We found that the stemness genes such as SOX2, POU5F1, WNT3A and TERT were down regulated up to seven fold in patients with non-healing wound. In particular, MSC specific markers and associated genes such as BGLAP, NGFR, EGF, HNF1A, IFNG and BGLEP were down regulated four to seven fold in comparison to patients with a healing wound. We have additional evidence showing that the stem cell signaling factor Wnt3a is down regulated in impaired-healing wounds in an animal model which correlate to our clinical findings. Defining the repertoire of stem cell and stem cell related signals expressed in patients with chronic non-healing wounds will help define the environmental niche of non-healing wounds relative to normal healing. These data will contribute in identifying target cells, genes and/or molecules to promote effective treatment of chronic non-healing wounds.

Poster Board Number: T-3344

REAL TIME BIODISTRIBUTION DETERMINATION OF NONCULTURED ADIPOSE-DERIVED REGENERATIVE CELLS AFTER INTRAVASCULAR INJECTION INTO A HEALTHY SYNGENEIC HOST ANIMAL

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Understanding the temporal and anatomical patterns of biodistribution by a cellular therapeutic is critical for establishing its safety profile. Biodistribution is dependent on many different variables such as cell type and origin, health status of the recipient, and route of delivery. Therefore, where and for how long the cells will reside is likely unique for each combination of variables. Establishing an understanding of how the cells behave when delivered into a normal, healthy recipient provides a baseline for comparison and helps address one of the key cell therapy safety questions. "What happens if I inject the cells into someone who does not need them?" Materials

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and Methods 10 normal healthy FVB/N strain female mice were recipients of adipose-derived stem and regenerative cells (ADRCs) delivery at three different doses (2.5, 4 and 6 x 10^6 cells per animal) by tail vein injection. ADRCs were isolated from inguinal fat pads of 50 syngeneic donor animals transgenic for the luciferase gene. After mincing fat pads, adipose was treated with Celase® reagent to enzymatically release cells. Cells were concentrated by centrifugation and then resuspended to delivery doses. Luciferase-positive ADRCs were tracked using real time assessment of luciferase activity with the Xenogen™ system 2 hours, and then 1, 2, 3, 7, 10, 14, and 21 days after injection. Dorsal, lateral and ventral images of cell distribution were captured and assessed semi-quantitatively. Results The distribution pattern of ADRCs was similar regardless of dose. Upon tail vein injection ADRCs were found within the lungs and spleen by two hours. By day 3 cells were observed still primarily within the lungs and spleen but significant relocation to the mandibular and parotid lymph nodes had occurred. No signal activity was observed in the brain, heart or other major organ systems. Peak luciferase intensity occurred between initial the time point of 2 hours post-injection and seven days, depending on the animal, but did not correlate strongly with dose in the range delivered. By day 21, only background signal was detectable. Conclusion This study reports the results of the first ever GLP-level study of ADRC biodistribution. Murine ADRCs home into the spleen, lymphatic system after initially being filtered out by the pulmonary capillary bed in healthy, non-injured, syngeneic animals. Both the pattern and temporal retention of the cells is consistent with a normal physiological response to syngeneic cells and is consistent with the conclusion that vascular delivery of these cells is safe. Importantly, these data provide a baseline to which variations in cell distribution and retention may be compared in various injury and disease models.

Poster Board Number: T-3345

ENHANCEMENT OF CUTANEOUS WOUND HEALING BY HUMAN ADIPOSE-DERIVED STEM CELLS: THE EFFECT OF SHORT TERM SPHEROID FORMATION

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Adipose-derived stem cells (ASCs) represent an important source of mesenchymal stem cells because abundant autologous cells can be easily obtained via liposuction procedures. ASCs are commonly cultured as monolayers using conventional tissue culture techniques. These methods have been proved adequate, but several reports have demonstrated a loss of the replicative ability, colony-forming efficiency, and differentiation capacity with time in culture. In this study, we tested the hypothesis that ASCs experiencing short-term spheroid formation exhibit enhanced therapeutic potentials, which can accelerate cutaneous wound healing in a nude mice model. The ASCs were passaged 2 times and plated onto chitosan films for spheroid formation. After 7 days, the spheroids were dissociated and replated on tissue-culture polystyrene. Culture media was refreshed every 2-3 days. ASCs were cultured for 7 more days and then harvested for experiments. ASCs that were constantly cultured on tissue-culture polystyrene were used as the control. Comparing to monolayer culture, spheroid-derived ASCs shared similar distribution of cell surface markers, and their adipogenic and osteogenic differentiation capabilities were comparable. Lactate dehydrogenase assay revealed less cytotoxicity of spheroid-derived ASCs under a serum starvation condition. Moreover, spheroid-derived ASCs exhibited higher expansion efficiency and colony-forming activity. Importantly, we demonstrated that spheroid-derived ASCs exhibited significant upregulation of CXCR4, hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), and pluripotency marker genes (Sox-2, Oct-4 and Nanog). The differences in protein expression were further confirmed by western blot analysis. Spheroid-derived ASCs also showed significant more migration in an *in vitro* wound healing assay and a transwell migration assay, but the enhanced migration could be inhibited by a CXCR4-specific peptide antagonist (AMD3100). The enhanced expression of matrix metalloproteinases (MMP-9 and MMP-13) in spheroid-derived ASCs could also be inhibited by AMD3100. By application of the expanded ASCs in a dorsal cutaneous wound model of nude mice, we further showed a faster wound healing in the group that received spheroid-derived ASCs at postoperative day 10. In the cutaneous wounds that received spheroid-derived ASCs, histology showed a significantly thicker epidermal layer and immunohistochemistry of human nuclear antigen demonstrated more cellular retention of ASC. Moreover, significantly more cells labeled with the proliferative marker Ki-67 were noted in the group of spheroid-derived ASCs. The results presented here indicated that ASCs can be activated nonchemically by short-term spheroid formation to express CXCR4, HGF, VEGF, and pluripotent markers. The upregulation of CXCR4 in the spheroid-derived ASCs further increases MMP-9 and MMP-13 expression, resulting in enhanced migration of these cells. The interaction between stromal-derived factor-1 and CXCR4 plays an important role in the homing of mesenchymal stem cells to the site of injury. In our animal study, we observed significantly more cellular engraftment of spheroid-derived ASCs in the cutaneous wound tissue with accelerated wound healing comparing to monolayer-cultured ASCs. Therefore, short-term spheroid formation of ASCs on chitosan films can increase their therapeutic potentials for wound healing.

Poster Board Number: T-3346

CONSTRUCTION OF LARGE AMOUNTS CULTURE SYSTEM BY AN AUTOMATED CELL PROCESSING MACHINE FOR THE CELL TRANSPLANTATION

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Recently, practical applications of pluripotent and/or multipotent stem cell for various diseases are proceeding exponentially. Several clinical trials has been reported a significant improvement of patient prognosis and quality of life using autologous cells. While they will widespread as the general health care, manufacturing cellular products remains to be solved. We are conducting a clinical trials using autologous cardiac stem cells (CSCs) from patients with ischemic heart disease (AutoLogous Human CArdiac-Derived Stem Cell to Treat Ischemic cArdiomyopathy, ALCADIA). The phase I trial is approaching to complete, and the phase II multicenter trial is under a plan. In order to perform the phase II trial effectively, it's necessary to develop a system to cultivate stem or progenitor cells with a given quality and high throughput. To solve this problem, we focused on the robotics of automated and high throughput culture system (AutoCulture) made by Kawasaki Heavy Industries. AutoCulture can change the medium, centrifuge, split, and record the morphology, and has already succeeded in culturing the cell lines such as HeLa cell, and can cultivate the maximum 29 types of cell lines at the same time. However, it has not been reported stem

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or progenitor cells have been cultivated for clinical application by a automated robotics in Japan. Therefore, we have examined and compared the growth rate and the characteristics of CSCs cultivated by automated cell processing machine and the manual handling culture. In this study, there were no significant difference of CSCs growth rates between the AutoCulture (Population Doubling: PD+6 to 7 in 2 weeks) and the manual handling culture (PD+6 to 7 in 2 weeks).

Poster Board Number: T-3347

INFLAMMATORY STATUS DETERMINES THE EFFECTIVENESS OF MESENCHYMAL STEM CELL THERAPY OF ADVANCED LIVER CIRRHOSIS

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Mesenchymal stem cells (MSCs) have been shown to be effective in the treatment of inflammatory disorders only in some patients. To investigate the factors that determine the effectiveness of MSC therapy, we established the mouse model of severe liver cirrhosis with carbon tetrachloride and found that MSCs were highly effective in reversing serum levels of total bilirubin, albumin, aspartate aminotransferase and alanine aminotransferase as well as liver fibrin deposition. Since our previous studies have shown that the immune modulatory effects of MSCs are elicited by inflammatory cytokines, we treated the cirrhotic mice with dexamethasone prior the administration of MSCs. We found that such pretreatment dramatically eliminated the effects of MSCs, revealing a critical role of inflammation in the curative effects of MSCs. Interestingly, in clinical settings, among the liver cirrhosis patients transfused with MSCs, those belonged to groups with Child-Pugh score B and C, especially patients with acute inflammation, exhibited improvement of their conditions. Our findings demonstrate that the inflammatory status determines the effectiveness of MSCs in treating liver cirrhosis and provides a novel strategy for patient selection and treatment protocol designs for mesenchymal stem cell therapy.

Poster Board Number: T-3348

LOW SERUM CULTURED ADIPOSE-DERIVED MESENCHYMAL STEM CELLS AMELIORATE CRESCENTIC GLOMERULONEPHRITIS BY FUNCTIONAL POLARIZATION OF MACROPHAGES INTO IMMUNOREGULATORY M2 PHENOTYPE

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Introduction: Glomerular crescents determine renal prognosis in patients with rapidly progressive glomerulonephritis, however efficient therapeutic approach to regulate crescent formation has not emerged since diverse leukocyte subsets contribute to the lesion. We have reported that adipose tissue-derived stem cells (ASC) promoted regeneration in a rat model of acute kidney injury. More recently, we have shown that ASC more strongly modulate T-cell immune reaction than bone marrow derived mesenchymal stem cells (BM-MSC). In the present study, we examined the renal protective effects of ASC focusing on their immunomodulatory properties in anti-glomerular basement membrane glomerulonephritis (anti-GBM GN) leading severe crescentic formation. Methods: Necrotizing crescentic glomerulonephritis was induced in WKY rats by intraperitoneal injection of anti -GBM mAb, which bore close resemble to nephritis in human Goodpasture disease. Renal function and histology were assessed in animals treated with ASC or BM-MSC. To evaluate ASC-driven functional M2 polarization in macrophage, we cultured peritoneal macrophages with ASCs or BM-MSC. Results: Intravenous injection of ASC significantly prevented renal dysfunction and proteinuria in diseased animals. The score of crescent formation was significantly decreased in ASC group compared to control group. Interestingly, infiltration of M2 macrophages in glomeruli was increased only in ASC group despite comparable number of infiltrated macrophages to control group. IL-10 concentration in renal cortex from diseased rat was higher in ASC group than in control group. *In vitro* co-culture system clearly demonstrated that ASC, but not BM-MSC, directly turned macrophage into M2 phenotype. Moreover, these effects of ASC were more prominent in low serum cultured ASC (LASC) than high serum cultured ASC (HASC). Administrated ASCs were scattered in multiple organs and number of ASC in diseased glomeruli was 1.5/glomerulus/cross section without any difference between HASC and LASC, but this would be efficient since individual LASC could polarize around 200 macrophages to M2 phenotype *in vitro*. These results collectively suggest that LASC recruited into diseased glomerulus make stronger effect on macrophages for protection of glomerular injury and crescent formation than HASC. Conclusion: ASC exerted profound immunoregulatory properties especially on macrophages and ameliorated glomerular injury in rat anti-GBM glomerulonephritis model. In human immunosuppressive therapy such as corticosteroid and cyclophosphamide remains the prevailing therapy for CGN, but this is often restricted for their adverse effect such as infection and agent cytotoxicity. Therefore, LASC administration would be desirable therapeutic approach to improve prognosis of anti-GBM GN patients.

Poster Board Number: T-3349

ASTROGLIAL ACTIVATION BY ENVIRONMENTAL ENRICHMENT SYNERGISTICALLY IMPROVES FUNCTIONAL RECOVERY BY TRANSPLANTED MESENCHYMAL STEM CELLS

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We investigated the effects of environmental enrichment (EE) on the function of transplanted mesenchymal stem cells (MSCs) and the combined effect of EE and MSC transplantation on neurobehavioral function in an animal model of chronic hypoxic-ischemic (HI) brain injury. HI brain damage was induced in 7-day-old mice by unilateral carotid artery ligation and exposure to hypoxia (8% O2 for 90 min). At 6 weeks of age, the mice were randomly injected with either MSCs (1×105 cells) or PBS into the striatum and were randomly assigned to either EE or standard cages (SC), comprising MSC-EE (n=18), MSC-SC (n=19), PBS-EE (n=12), PBS-SC (n=17), and untreated controls (n=23). Rotarod, forelimb-use asymmetry, and grip strength tests were performed to evaluate neurobehavioral function. The fate of transplanted cells and the levels of endogenous neurogenesis, astroglial activation and paracrine factors were also measured. As a result, EE and MSC transplantation synergistically improved rotarod latency at constant speed of 48 rpm (156.8±18.1 sec) and at accelerating speed (168.5±15.1 sec) 8 weeks after treatment ($p<0.05$). Forelimb-use asymmetry and grip strength were also improved compared to those of the other group ($p<0.05$), whereas ASC transplantation causes only hemiplegic grip strength recovery. MSC-EE mice also showed a higher GFAP+ cell density than the other groups at 2 weeks post-treatment ($F = 18.186$, $p<0.05$); GFAP+ cell densities were MSC-EE (12.5 ± 1.2 %), MSC-SC (8.2 ± 0.7 %), PBS-EE (5.7 ± 0.5 %), and PBS-SC (4.9 ± 0.6 %). At 8 weeks after treatment, MSC-EE (5.5 ± 0.7 %) still showed a sig-

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nificant increase in GFAP+ cell density compared with that of PBS-SC (3.2 ± 0.5 %) ($F = 3.047$, $p<0.05$). However, the levels of the glial scar marker CS-56 did not differ among the groups, demonstrating that the combination of EE and MSCs do not increase the detrimental glial scar formation which inhibits neuroregeneration after damage. Among paracrine factors, the level of FGF-2 was significantly elevated in mice with EE after transplantation of MSCs (908 ± 162 pg/ml) compared with those in MSC-SC mice (350 ± 38.2 pg/ml), PBS-EE (174 ± 37.1 pg/ml), and PBS-SC mice (148 ± 36.5 pg/ml) at 2 weeks after treatment ($F = 11.249$, $p<0.05$). Furthermore, this FGF-2 upregulation was sustained until 8 weeks after treatment in mice with EE after transplantation of MSCs (970 ± 240 pg/ml) compared to those in MSC-SC mice (406 ± 105 pg/ml), PBS-EE (373 ± 116 pg/ml), and PBS-SC mice (307 ± 69.2 pg/ml) ($F = 4.633$, $p<0.05$). In conclusion, EE and MSC transplantation synergistically improved neurobehavioral functions. The underlying mechanisms of this synergism included enhanced repair processes such as astroglial activation coupled with upregulation of FGF-2. Acknowledgment This study was supported by grants from the National Research Foundation (NRF-2010-0020408; NRF-2010-0024334; SC-4160) and the Yonsei University College of Medicine (6-2011-0078).

Poster Board Number: T-3350

MITIGATION OF LETHAL SYSTEMIC RADIATION EFFECTS WITH DELIVERY OF PLACENTAL ADHERENT 3D GROWN STROMAL CELLS (PASC)

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A very limited arsenal of treatments is available for the salvation of individuals exposed to lethal dose of ionizing radiation. Placental cells trans-differentiation potential is limited. Nevertheless, they secrete a variety of cytokines and may be involved in the regulation of the immune response to enhance tissue repair. The current study used placental cell preparation that were expanded in a special 3D bioreactor system (PLX cells) to alleviate systemic radiation damage. C3H mice were exposed to total body lethal radiation dose of 850cGy. IV injection of the placental cells 24hrs post irradiation resulted in a ~3 fold elevation of their survival rate. The highly statistically significant effect was associated with mitigation of systemic radiation effects, as manifested by an elevation of the bone marrow counts during the recovery period and elevated peripheral blood cells profile. This allowed the irradiated mice overcome the radiation induced bone marrow syndrome. The xenogeneic human placental cells tend to reside transiently for up to 3-4 days in the recipient's lungs after IV injection while excreting their effect during this short time interval. No complications associated with the subsistence of the placental cells for this time interval were observed. In IM injection a much higher number of cells could be safely injected. Injection of $2x10^6$ cells at day 1 and 5 after irradiation by 770cGy reduced the radiation induced mortality by almost 4 fold. We propose that the promising use of 3D grown placental cells for alleviation of radiation effects may set a basis for a new easily available simple allogeneic cell therapy for critical radiation exposure following nuclear disasters or radiation accidents. The placental cells for such treatments could be available as commercial allogeneic frozen cells product "off the shelf" and could be effective when injected to suspected victims even a day or more after their high dose radiation exposure. This renders such placental cell treatment a potential revolutionary "off the shelf" treatment for individuals as well as mass populations exposed to high heterogeneous doses of radiation. Further studies may examine the potential use of placental cells for clinical conditions associated with severe complications due to the use of cytotoxic-drugs and radiation therapies. Investigation of the mechanism behind the effects of the PLX cells administration and their vast effect in mitigation of radiation effect are on-going.

Poster Board Number: T-3351

THE EFFECTS OF MESENCHYMAL STEM CELLS ON OSTEOGENESIS AND ANGIOGENESIS OF HYDROXYAPATITE SCAFFOLD

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One of the major challenges in bone tissue engineering is the constructs vascularisation ability. One of the current strategies is scaffold's design with linear hole. mesenchymal stem cells (MSCs) were differentiated from bone marrow of Wistar rat and their phenotypes were proved before implantation. . The porous hydroxyapatite scaffolds were synthesized by casting removal method. . In group A, scaffolds were seeded with MSCs. Free scaffolds were identified as a control group. In each group sampels in the time order of fourtheen; and 21 days were sent for histological analysis. . In comparison, the seeded grafts results in a higher angiogenesis and osteogenesis factors. The scaffold with linear hole and seeded cells may improve neovascularisation in grafts and improved osteogenesis. This method may be a useful strategy to facilitate bone regeneration.

Poster Board Number: T-3352

VITAMIN C ASSISTS ADIPOSE-DERIVED STEM CELL TRANSPLANTATION IN MUSCLE INJURY MODEL

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Stem cells have been widely used to cure intractable diseases. Senile muscle atrophy and hereditary muscle dystrophies are hard to cure, and muscular regeneration after damages is usually imperfect because of the development of fibrotic tissues. Therefore, stem cell transplantation has been an effective therapeutic trial to regenerate damaged skeletal muscles. Various types of stem cells have been used in intramuscular transplantation, we used adipose-derived stem cells (ASCs) in the present study based on following reasons; First, ASCs can differentiate into multiple types of cells including satellite cells and myocytes, which are directly needed for muscle regeneration, and endothelial cells, which can support muscle regeneration by supplying adequate blood flow to the damaged area; Second, ASCs also can be obtained easily in large amounts by lipectomy; Finally, the most critical benefit of ASCs is that they are considered to have immunomodulatory properties, and consequently, allogenic ASCs can be transplanted with less concern of major histocompatibility complex incompatibility between donor and recipient. However, stem cells transplantation is not a master determinant. In other words, if transplanted cells cannot survive, they cannot play their own role in muscle regeneration. Thus, we hypothesized that vitamin C which can enhance the viability of the transplanted stem cells via its potent anti-oxidative effects would increase the therapeutic efficiency. Senescence marker protein 30 (SMP30) knock-out (KO) mice were used in the experiment since SMP30 KO mice cannot synthesize vitamin C for themselves. Mice were injured their central part of the gastrocnemius muscle by a surgical muscle laceration. Vitamin

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C was depleted or provided in drinking water *ad libitum* considering experimental groups and allogenic adipose-derived stem cells were locally transplanted to the injured lesion. Vitamin C supplied mice exhibited more complete regeneration of damaged muscle than those depleted vitamin C in both gross and microscopic observations. Carbonylated proteins, which are the end-products of oxidative stress, were detected potently in vitamin C-depleted mice and the positivity was more potent in the presence of transplanted stem cells. Moreover, the serum total vitamin C level and ascorbic acid (reduced form) to dehydroascorbic acid (oxidized form) ratio also were decreased in the ASC transplanted groups. Collectively, these data can be considered as proof of vitamin C utilization by cells *in vivo*. In conclusion, we demonstrated that vitamin C increases the viability of transplanted stem cells by diminishing the oxidative and inflammatory stress, and thereby it can positively affect muscle regeneration in the case of adipose-derived stem cell transplantation and might be used as a therapeutic agent supplied in cell transplantation.

Poster Board Number: T-3353

ENHANCED HOMING, PERMEABILITY, AND RETENTION OF BONE MESENCHYMAL STEM CELLS (MSC) USING PULSED FOCUSED ULTRASOUND: IMPLICATIONS FOR TARGETING STEM CELL DELIVERY

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Introduction: Therapeutic bone marrow stromal cells (BMSC, also known as mesenchymal stem cell, MSC) are capable of homing to sites of inflammation and ischemia following intravascular injection, but are often limited by inefficient tropism to pathological sites. Directed homing and tissue integration of BMSCs may be key to improving their therapeutic applications. Therefore the development of a non-destructive, non-invasive technique that would release chemoattractants resulting in BMSCs localization within or at the periphery of pathology would be valuable for cellular therapies. Pulsed focused ultrasound (pFUS) is an emergent noninvasive modality that non-destructively emphasizes the non-thermal effects of FUS (i.e., acoustic cavitation and radiation forces). These non-thermal mechanisms increase tissue permeability and enhance drug or gene delivery, while inducing only minor and transient morphological changes within the treated region. Relatively little is known about the cellular and molecular biological effects of pFUS exposures beyond the structural changes that result in vascular leakage. Cellular and molecular biology of tissues can be dramatically altered by mechanical force and stress through the process of mechanotransduction (i.e. tissue cytokine and chemokine expression in response to pFUS mechanical forces). In this study, we examined the bioeffects of pFUS in murine muscle and kidney tissue and found that pFUS exposures induced local expression of chemoattractive factors (cytokines, growth factors, integrins) and investigated the utility of pFUS to direct BMSC homing *in vivo*.[[Unsupported Character -  ]]Methods: Balb/c or C3H mice were administered pFUS to the leg or kidney. Some mice were intravenously administered 106 human BMSC 2 hr post-pFUS. Mice with renal disease received cisplatinum (15 mg/kg) induced acute tubular necrosis (ATN) 24 hr before pFUS. At various time points, tissue was subjected molecular analyses for cytokine and growth factors, histology, or immunofluorescence for detection of BMSC. Results: pFUS significantly increased local cytokines (i.e., IL1β, IL-2, IL-3, IL-5, IL-6, IL-10, IL-17, IFNγ, MCP- 1, GMSCF, and RANTES) in pFUS-treated tissue that declined to contralateral levels by 3 days post-pFUS. Elevated cytokines were accompanied by increased expression of growth factors (i.e., VEGF, FGF, PlGF, HGF SDF-1) and ICAM and VCAM usually associated with active BMSC homing. Following pFUS, significantly greater numbers (5-10x) of IV BMSC marginated into pFUS-treated tissues compared to controls. Enhanced homing, permeability, and retention (EHPR) of BMSCs were significantly increased (10x) by repeated doses of BMSC coupled with repeated pFUS exposures in muscle without damage to tissue. pFUS coupled with BMSC infusion in the ATN model resulted in significantly greater numbers of stem cells in treated kidney compared to control and was also found to decrease the peak BUN and Creatinine level on day five post cisplatinum injection compared to control mice or mice that received only BMSC. Conclusions: The ability to couple pFUS with cell infusions, both during the innate pathological inflammation or after it has resolved, greatly increases the flexibility of stem cell-based therapies. pFUS may provide the temporal and spatial control over directing stem cells to sites of pathology thereby potentially improving treatment of pathologies or regenerative medicine.

Poster Board Number: T-3354

INJECTABLE BIOMATRIX FOR ARTHROSCOPIC SURGERY IN DEGENERATIVE CARTILAGE

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Abrasion, microfracture and osteochondral graft are examples of methods for treating osteoarthritis. However, these procedures are invasive, requiring open surgery. Herein, we have developed an injectable biomatrix (IBM) which can be applied on damaged or degenerative cartilage. We have also demonstrated the efficacy of the IBM with umbilical cord-derived mesenchymal stem cells (UC-MSCs) on damaged rabbit cartilage. The IBM was composed of 2% hyaluronic acid derivative and 3% type I collagen isolated from human umbilical cord at a ratio of 1 : 2. UC-MSCs within the IBM were successfully differentiated into chondrocytes *in vitro* and *in vivo* conditions through transplanting to the subcutaneous layer of nude mice. For efficacy test, UC-MSCs within IBM were injected into the injured cartilage of adult New Zealand white rabbits. After 12 weeks of transplantation, histological analysis showed the regeneration of cartilage on the damaged site. In conclusion, our study shows that IBM has promising effects for the treatment of degenerative osteoarthritis via arthroscopic procedures.

Poster Board Number: T-3355

PRECLINICAL AND CLINICAL TRIALS OF BONE-MARROW MESENCHYMAL STROMAL CELL IN AMYOTROPHIC LATERAL SCLEROSIS

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Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease characterized by motor neuron loss. Although the underlying cause of the disease remains unclear, a variety of pathogenic mechanisms have been proposed. Despite promising preclinical studies showing the modification of the disease progression, most trials have failed to demonstrate any significant improvement in outcome. Therefore, stem cell therapy has been proposed as an alternative therapy for ALS. In animal model of ALS (transgenic SOD1m mice), we evaluated the dose-dependent effects of human bone marrow mesenchymal stem cells (hMSCs)

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obtained from an ALS patient (ALS-hMSCs) on SOD1 mice via intrathecal injection and showed the practical possibility of it for hMSCs. We transplanted different doses (1×10^4, 2×10^5, and 1×10^6) of ALS-hMSCs into the cisterna magna and performed clinical observations including symptom onset and survival time, and locomotor performance using the rotarod test. A cell dose of 1×10^6 cells significantly prolonged life span and delayed the decline of motor performance. Most injected hMSCs distributed in the ventricular system and subarachnoid space and some migrated into the brain and spinal cord. These data suggested that intrathecal injection with an optimized cell number might be the practical potential route for stem cell therapy in ALS. With animal data, we evaluate efficacy and safety of Autologous Bone Marrow-derived Mesenchymal Stem Cell treatment in patients with ALS. After a lead-in period for 3 months, 22 patients were treated with MSCs twice at an interval of 1 month. After initial MSCs injection, all patients were followed up for 3 months and their disease course, clinical characteristics were assessed. Disease status of patients were analyzed with ALS functional rating scale-revised (ALSFRS-R) for primary outcome measure, and additional clinical findings after treatment were all collected for secondary outcome measure and safety. Age and disease-duration matched patients with ALS were selected as a control group. During the follow-up period, MSCs treatment yielded a significant lesser change of ALSFRS-R score, compared to control group (1.54 vs 3.56, $p<0.01$). Moreover, the slop of decline of ALSFRS-R was significantly lower during the follow-up period, compared to the lead-in period in MSCs treatment group (2.68 vs 1.54, $p=0.04$), whereas the slopes during the two periods were not different in the control group (3.15 vs 3.56, $p=0.37$). MSCs treatment was well tolerated except for occurrences of transient headache, low back pain, and myalgia. Our results suggest that intrathecal MSCs injection can slow disease progression and might be used as a disease modifying modality as an alternative treatment choice in patients with ALS

Poster Board Number: T-3356

MESENCHYMAL STEM CELL EXOSOME: A NOVEL STEM CELL-BASED THERAPY

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Mesenchymal stem cells (MSCs) are the cells of choice for regenerative medicine with more than 100 registered clinical trials evaluating their efficacy in 2010. The main allures of MSCs lie in their protective effects on an amazingly wide spectrum of tissue injury, ease of isolation from ethically palatable adult tissue sources, a large *ex vivo* expansion capacity, as well as demonstrated multipotency and immunosuppressive activity. It has been increasingly observed that the therapeutic efficacy of MSC therapy is not dependent on the engraftment of MSCs at the site of injury or differentiation capability of the transplanted MSC. To reconcile the discrepancy between these observations and the therapeutic effects of MSC, it was postulated that transplanted MSC secrete trophic mediators that reduce tissue injury and enhance repair. Consistent with this, we demonstrated that MSCs secrete membrane vesicles known as exosomes and these exosomes reduce myocardial ischemia/reperfusion injury in a mouse model. The use of secreted exosomes and not MSCs eliminates the need for transplantation of viable replicating cells and thus mitigates many of the associated safety concerns and limitations. MSC exosome contains protein and RNA. Proteomic analysis of the exosome proteome revealed a diverse array of proteins distributed over a wide range of biochemical and cellular processes, and thus a large potential to intervene in a wide range of cellular activities. This large potential could provide a mechanistic underpinning for the therapeutic effects of MSC on a wide spectrum of complex diseases. Therefore exosomes represent a novel stem cell-based therapy.

Poster Board Number: T-3357

ASSESSMENT OF THE SAFETY TRANSPLANTATION OF ALLOGENEIC MESENCHYMAL STROMAL CELLS OF BONE MARROW IN PATIENTS INFLAMMATORY BOWEL DISEASES: 4 YEARS OBSERVATION.

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Patients suffering from long-term Inflammatory Bowel Diseases (IBD) are at increased risk for cancer of the intestine (7-10 times more frequently than in the general population of people). IBD patients who are suffering from more than 10 years, the probability of cancer is increasing annually at 0.5%. Cell therapy is a medical technology a high degree of risk. In addition to invasive methods for early diagnosis of cancer, determination of tumor markers can identify groups at increased risk of cancer. Aim. To evaluate the safety of transplantation of allogeneic mesenchymal stromal cells (MSCs) of bone marrow in the treatment of patients with Ulcerative Colitis (UC) and Crohn's disease (CD). Materials and methods. In the Department of Pathology bowel 86 patients with UC and CD in 2008-2012 years a behavioral system transplantation of allogeneic MSCs. 68 patients has a fore -year observation period. Patients underwent endoscopic control study of colon with multiple ladder biopsy. In the serum of patients determines the levels of tumor markers CA 242, CA 19-9 and REA as well as transforming growth factor-1β (TGF-1β) using test-systems company "Protein contour" (St. Petersburg), Orgentec (Germany) Evroimmun (France). Results. In 31 patients with UC for 12 months of observation, a decrease of endoscopic activity index from 8,3±0,26 to 0,76±0,2 points. In 8 patients with UC of 12 (66.7%) recorded the clinical and morphological remission. Baseline tumor marker CA 242 to MSC transplantation was 5,26±0,2 U/ml, the level of tumor marker CA 19-9 - 5,03±0,18 U/ml. After 6 months the level of tumor marker CA 242 was 4,9±0,17 U/ml, CA 19-9 - 4,83±0,16 U/ml after 12 months - 4,8±0,17 U/ml 4,96±0,16 U/ml, respectively. After 24 months the level of tumor marker CA 242 was 4,85±0,2 U/ml, CA 19-9 - 5,25±0,22 U/ml. After 48 months the level of tumor marker CA 242 was 4,95±0,2 U/ml, CA 19-9 - 5,65±0,22 U/ml, REA - 6,25±0,25 U/ml. The level of TGF-1β before MSC transplantation was 73,4±16,2 pg/ml, rising to 1 week after injection to 792,5±50,5 pg/ml at 4 weeks after transplantation of MSCs level of TGF-1β was - 448,6±76,1 pg/ml. These levels of TGF-1β (less than 1500 pg/ml) are safe in terms of tumor development (malignancy) and maintenance of tumor growth or metastasis. During 24-46 months (42,3±0,8) of observation for patients who carried MSC transplantation, in neither case has been registered increase of tumor markers of over 10 U/ml and TGF-1β levels above 1500 pg/ml, as well as malignant transformation of IBD according to endoscopic examinations. Conclusion. Transplantation of allogeneic mesenchymal stromal cells of bone marrow did not lead to the development of tumor process and is a safe method of biological therapy of IBD.

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Poster Board Number: T-3358

MESENCHYMAL STEM CELLS DELIVER SYNTHETIC MICRORNA MIMICS TO GLIOMA CELLS AND GLIOMA STEM CELLS AND INHIBIT CELL MIGRATION AND SELF-RENEWAL

Lee, Hae Kyung[1], Finniss, Susan[1], Cazacu, Simona[1], Bucris, Efrat[2], Ziv-Av, Amotz[2], Xiang, Cunli[1], Bobbit, Kevin[3], Rempel, Sandra A.[4], Mikkelsen, Tom[1], Slavin, Shimon[5], Brodie, Chaya[6]

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Malignant glioma are the most common and aggressive primary brain tumors with poor prognosis and median survival of 12-14 months. Limitations to therapy include the distinctly infiltrative nature of the tumors and the high resistance to radio- and chemotherapy of residual tumor cells and glioma stem cells (GSC). MicroRNAs are emerging as potential cancer therapeutics; however, their clinical use is hindered by a lack of effective delivery mechanisms to tumor sites. Mesenchymal stem cells (MSCs) have been recently shown to migrate to experimental glioma and to exert anti-tumor effects by delivering cytotoxic compounds. Here, we examined the ability of MSCs derived from bone marrow, adipose tissue, placenta and umbilical cord to deliver synthetic microRNA mimics to neighboring glioma cells and glioma stem cells (GSCs). For these studies we examined the delivery of miR-124 and miR-145 mimics since both glioma cells and GSCs express very low levels of these miRNAs. Using fluorescent labeled miRNA mimics and in situ hybridization, we demonstrated that the MSCs examined delivered miR-124 and miR-145 mimics to co-cultured glioma cells and GSCs via gap junction-dependent and independent processes. Moreover, the transferred miR-124 mimic significantly decreased the expression of the target gene SCP-1 and its 3'-UTR tagged to luciferase. The delivered miRNA mimics also decreased the migration of glioma cells and the self-renewal of GSCs. These results suggest that MSCs can functionally deliver synthetic miRNA mimics to glioma cells and GSCs and may provide an efficient route of therapeutic miRNA delivery *in vivo*.

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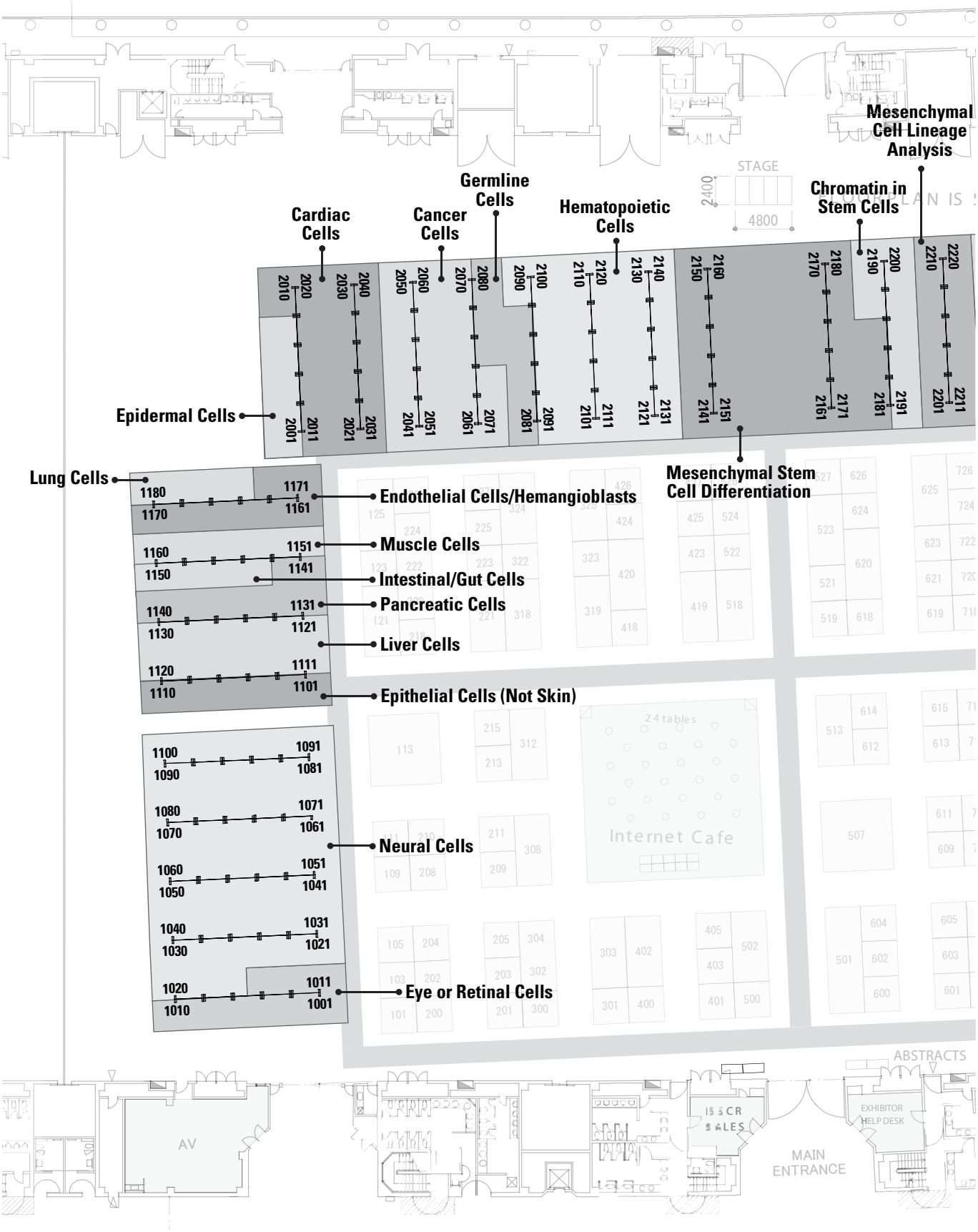

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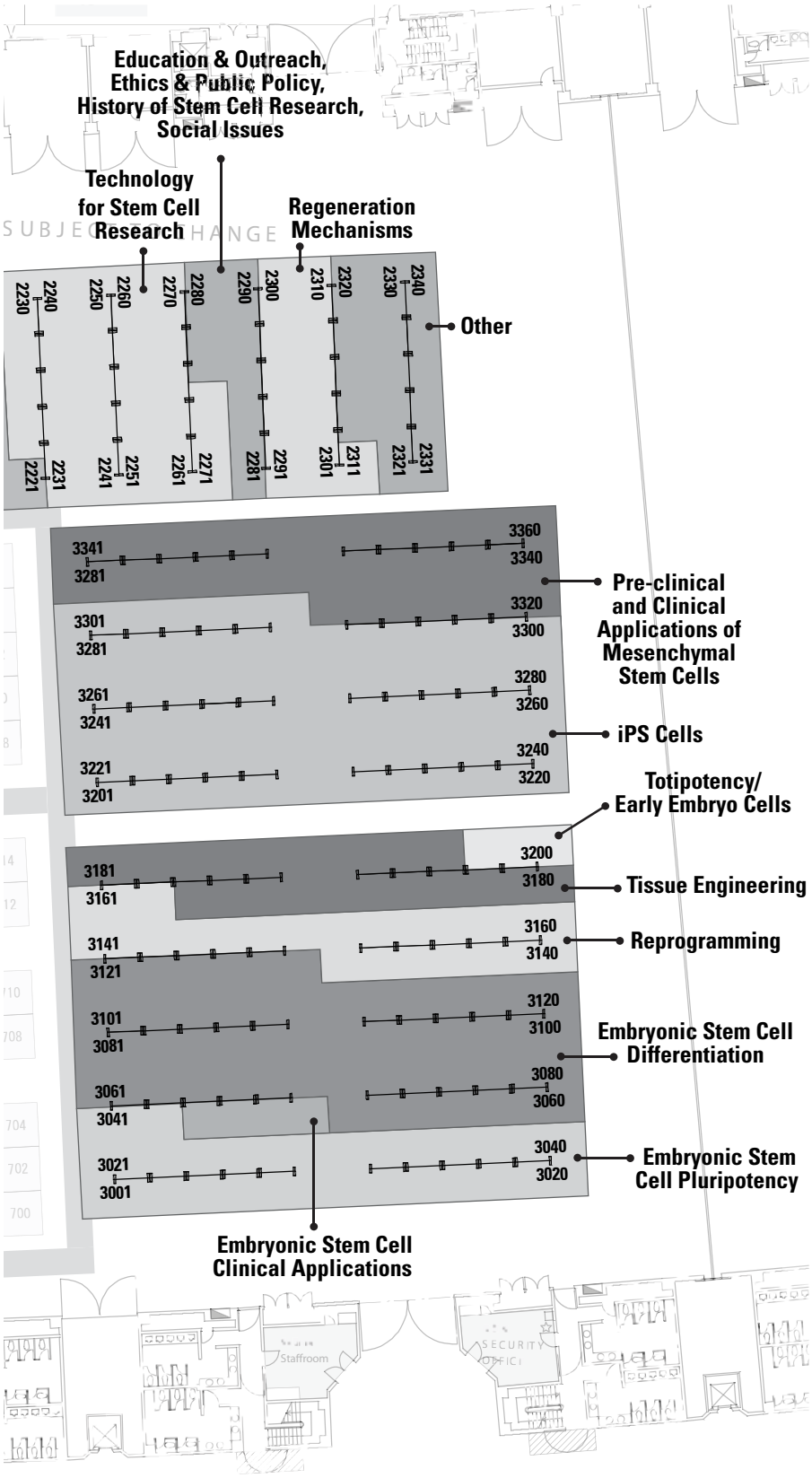

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NOTE: The presenters of the **ODD-numbered** posters are asked to present for the first hour of the receptions. The presenters of the **EVEN-numbered** posters are asked to present for the second hour of the receptions.

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Eye or Retinal Cells

Poster Board Number: F-1001

A SCALABLE PROCESS FOR THE GENERATION OF RETINAL PIGMENTED EPITHELIUM CELLS FROM HUMAN PLURIPOTENT STEM CELLS IN DEFINED CONDITIONS

Maruotti, Julien, Wahlin, Karl, Gorrell, David, Zack, Donald J.

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Age-related macular degeneration (AMD) is the most common cause of irreversible visual loss in the elderly in the Western world. It is a devastating disease of the retina that can lead to central blindness. It affects the retinal pigment epithelium (RPE) - the layer of cells that surrounds and nourishes the neurosensory retina. When RPE cells do not function properly, photoreceptor cells begin to die and vision is lost. Currently, there are few if any available options to treat or replace diseased RPE. Cell-based transplantation strategies offer the promise of being able to restore RPE cells, thus potentially limiting vision loss. Therefore, establishing an abundant and quality source of donor cells is of utmost importance. Human pluripotent stem cells (hPSCs) may prove suitable for this purpose: significant advances have recently been made in inducing the differentiation of hPSCs toward an RPE-like cell fate and a Phase I clinical trial using such differentiated cells is currently ongoing. Nevertheless, and despite some improvements in term of differentiation efficiency, protocols described so far still require labor-and-time consuming manual steps for the propagation of hPSC, and for the isolation of RPE cells. We therefore sought to set up a simple protocol for the large scale production of RPE cells from hPSC. Human ESC and iPSC lines were cultured by clonal propagation on matrigel or synthemax using a ROCK pathway inhibitor: after more than 20 passages, the cells retained a high level of pluripotency (>95% OCT4+ and >80% TRA-1-60+ cells by flow cytometry) and maintained normal karyotypes. To initiate differentiation, cells were cultured in medium without pluripotency maintaining factors for about 50 days: many pigmented colonies were observed and more than 15% of the cells were positive for the RPE marker RPE65. The whole cell population was thereafter dissociated to a single cell suspension, and cultured for another 15-30 days: we observed that a majority of pigmented cells had a polygonal morphology, intermingled with a few fibroblast-like cells. The whole cell population was passaged a second time and purified using a negative selection with anti-fibroblast magnetic beads. This process led to a homogeneous layer of polygonal pigmented cells. These cells expressed key RPE markers by PCR and immuno-staining, and were polarized as evidenced by presence of Na+/K+ pump staining on their apical side and BEST1 staining on their basal side. hPSC derived RPE cells were able to phagocytose pH-rhodo labeled microbeads as well as rod outer segments. In addition, they secreted PEDF in the supernatant. Taken together, these data demonstrate that using our protocol hPSC derived RPE cells display many of the molecular and functional characteristics of endogenous RPE cells. From 2x10^5 starting hPSC (a single 35mm dish), it was routinely possible to generate at least 2x10^8 RPE cells (at second passage). In conclusion, we describe a simple protocol well suited for the large scale production of RPE monolayers in completely defined conditions.

Poster Board Number: F-1002

POLYCAPROLACTONE SCAFFOLD FOR DIFFERENTIATION OF HUMAN RETINAL PROGENITOR CELLS

Regatieri, Caio[1], Baranov, Petr[1], Melo, Gustavo[1], Stevanato, Lara[2], Patel, Sara[2], Sinden, John[2], **Young, Michael**[1]

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Purpose: Loss of photoreceptors due to retinitis pigmentosa, age-related macular degeneration and other age, trauma and genetic-related retinal degenerative disorders currently leads to incurable blindness. Since the regenerative capacity of human neural retina is highly limited, one viable treatment option is cellular replacement. Human retinal progenitor cells (RPC), isolated from the fetal retina, have been shown to form new functioning photoreceptors and restore retinal function following transplantation into retinal degenerative hosts. However, the low percentage of photoreceptors differentiating from the hRPC after subretinal transplantation limits translational studies.We hypothesize that the use of polycaprolactone scaffold for hRPC differentiation may significantly improve photoreceptor differentiation. Methods: hRPC were isolated from human neural retina at 18 week of gestational age and expanded under low-oxygen (3%) conditions up to passage 9. Karyotype analysis, immunocytochemistry and flow cytometry analysis for stemness, eye field, neuronal, proliferation and retinal cell markers were performed. The PCL film was prepared as previously described and coated with poly-L-Lysine and fibronectin. hRPC were seeded on the PCL film for 7 days. The resulting cell phenotype was assessed by immunocytochemistry and flow cytometry. The functional properties - by calcium imaging and explant assay were tested. hRPC, cultured on fibronectin-coated tissue-treated plastic were used as a control. Results: After differentiation on PCL, we observed a decrease in proliferative marker (PCNA, Cyclin D1) expression and an increase in photoreceptor marker expression. More than 50% of the cells express Opsin Blue, Opsin Red/Green or Rhodopsin. In standard culture conditions less than 5% of cells were found to express those markers. PCL differentiation also leads to an increase in number of cells integrated into retinal explants and in the number of cells responding to excitatory neurotransmitters. Conclusions: The differentiation of hRPC on PCL may be used to study retinogenesis (photoreceptor formation) *in vitro* and for pre-differentiation before transplantation.

Poster Board Number: F-1003

IDENTIFICATION OF CD 90 AND CD 105 POSITIVE MESENCHYMAL STEM CELLS IN THE ANTERIOR HUMAN LIMBAL STROMA

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INTRODUCTION: Human corneal epithelial stem cells are located in the basal layer of the limbus between the cornea and conjunctiva. These adult tissue-specific stem cells have the capacity to self-renew and generate functional differentiated cells that replenish lost cells throughout lifetime. Loss of limbal stem cells renders cornea with clinical disease called limbal stem cell deficiency. The maintenance, self-renewal and proliferation of stem cells must be regulated by a specialized microenvironment or niche which is thought to be located at the Palisades of Vogt and possibly involves limbal stromal cells, extracellular matrix components and

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several secreted factors. However, the nature of the limbal stem cell niche is not clearly understood. PURPOSE: In this study we intend to explore the cellular nature of the niche by understanding the distribution of mesenchymal stem cells in the human limbal stroma. METHODOLOGY: Serial 5 µm sections of human corneo-scleral segments from enucleated cadaver globes obtained from the Rotary Aravind International Eye Bank were immunostained for markers like CD 90, CD 105, Vimentin and K19. The expression of these markers was further evaluated on cells isolated from limbal and corneal stroma. RESULTS: CD 90 and CD 105 positive mesenchymal stem cells are present in the limbal anterior stroma, but not in the posterior limbal stroma. Such cells are also absent in the corneal stroma, thus indicating that these mesenchymal stem cells represent limbal stem cell niche. CK 19 was distributed in the basal cells of the limbus and some suprabasal clusters. Vimentin positivity was found in the limbal basal epithelium, corneal and limbal keratocytes. Isolated cells from limbal stroma showed positivity for CD 90, CD 105 and vimentin but were negative for K19 whereas corneal stromal cells were positive only for vimentin. CONCLUSION: Identification of CD 90 and CD 105 positive mesenchymal stem cells and understanding the factors secreted by them would help to develop better methods of generating stem cells in culture and thus favoring better treatment for patients with LSCD.

Poster Board Number: F-1004

DIFFERENTIATION OF HUMAN IPS INTO PHOTORECEPTORS INDUCED BY IPS -DERIVED PIGMENTED CELLS

Yue, Fengming[1], Tomotsune, Daihachiro[1], Ichikawa, Hinako[1], Yoshie, Susuu[1], Mogi, Akimi[1], Takahashi, Yuto[1], Masuda, Shoko[1], Shirasawa, Sakiko[2], Yokoyama, Tadayuki[2], Nagai, Mika[2], Sasaki, Katsunori[1]

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Photoreceptors cannot regenerate and recover their functions once disordered. In our previous research, retina cells were successfully induced from primate embryonic stem cells (ESCs) by co-culture with ESC-derived pigmented cells. In this study, we investigated induction of retinal pigmented epithelia (RPE) and photoreceptors from human iPS cells. Methods: RPE cells were derived from co-culturing iPS with Sertoli cells. After purified with density separation in a gradient of Percoll, RPE were co-cultured with iPS to induce photoreceptor differentiation. Pax6-GFP human iPS was constructed so that retina progenitors could be sorted by FACS. Results: RPE differentiation efficiency was significantly improved by nicotinamide treatment and co-culturing with Sertoli cells. The polygonal morphology with a compact cell-cell arrangement and pigmented cell relative gene or protein expressions indicated that these pigmented cells were RPE cells. 99% cells were proved to be RPE after purified using Percoll, Then these RPE were co-cultured with Pax6-GFP iPS. Retina progenitors were demonstrated with Pax6 gene by RT-PCR analysis. After sorting with FACS, Pax6-GFP positive cells were treated with retinoic acid (RA). The distinct increase of photoreceptor specific proteins and gene markers was found, such as CRX, IRBP, Rhodopsin, Rhodopsin kinase and Muschx10A. Besides, the differentiation of bipolar, horizontal cells was demonstrated by protein and gene expression. iPS cells which were co-cultured with RPE cells and treated with RA will be transplanted into the renal capsule of nude mice to examine whether Pax6-GFP-derived photoreceptors can organize into recipient tissue and the formation of teratoma could be avoided. Conclusion: These results indicate that co-culture of human iPS with iPS-derived RPE cells is a useful and efficient method for the induction of photoreceptors and provide an insight into the use of ESCs for retina regeneration.

Poster Board Number: F-1005

TRANSCRIPTIONAL REGULATION OF RETINAL FATE DETERMINATION FROM HUMAN INDUCED PLURIPOTENT STEM CELLS

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Development of the vertebrate eye is a complex process that is dependent upon the activity of numerous transcription factors. However, the process by which a retinal fate is specified from a primitive anterior neural progenitor cell remains largely elusive. Human induced pluripotent stem cells (hiPSCs) allows for the unique ability to recapitulate events during human development at stages that would otherwise be inaccessible to investigation. Building upon our previous studies, we sought to establish the role of key transcription factors during the establishment of a retinal fate. hiPSCs were directed to differentiate toward a retinal lineage using a targeted, stepwise differentiation process that mimics human retinogenesis. Experiments were designed to assess the developmental stages at which retinal cell fate determination was established from a primitive anterior neural population. Samples were collected every two days over the first twenty days of differentiation and gene expression analysis was performed via qPCR and immunocytochemistry. Following the identification of candidate transcription factors, these genes were tested for their ability to influence retinal fate determination through gene overexpression studies. From a primitive anterior neural population derived from hiPSCs, populations of retinal and forebrain progenitor cells could be readily identified within the first 20 days of differentiation. During this timecourse, retinal populations were characterized by the expression of key transcription factors which were absent from other non-retinal cell types. These candidate transcription factors were then cloned into lentiviral expression plasmids and cultures of hiPSCs were infected. The effects of overexpression of these genes were determined via qPCR and immunocytochemistry analyses to establish their ability to specify an early retinal fate. The work presented in this study helps to elucidate the mechanisms by which a retinal fate is specified from a more primitive population. The results of this study will assist in the establishment of efficient methods to generate retinal cells from hiPSCs and help establish these cells as a unique *in vitro* model system for studies of human development.

Poster Board Number: F-1006

A PILOT CLINICAL TRIAL: TRANSPLANTATION AND TRACKING OF HUMAN RETINAL STEM CELL IN OPTIC NERVE INJURY PATIENT

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The translational path for stem cells to the clinic is full of challenge. Traumatic optic nerve injury is one of the leading causes of incurable vision loss across the world. Neither pharmacological nor surgical interventions are significantly effective in reversing or halting the progress. Advances in stem cell sciences offer the hope for the patients with optic nerve injury. We investigated whether human retinal stem cells could be implanted into the patient's

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retina for treatment of vision loss caused by optic nerve injury. We isolated retinal stem cells (RSCs) deriving from the human embryonic eye tissues obtained from routine legal abortion under compliance with the ethical guidelines of the university. The RSCs generated relatively large cluster containing a mixture of cells. The stem cells were produced the different retinal cell types, demonstrating multipotentiality. The RSCs were implanted into the retina of the 7-day-old nude mice, positive cells could be observed in the vitreous layer, and migrating into the host retina and taking up residence within the cellular retinal laminae, including the outer nuclear layer, outer plexiform and inner nuclear layer. At 21 days post-implantation, intraretinal donor cells were found in 70% of the graft recipients. We then investigated the feasibility of implanting RSCs into the patient's retina and tracking them with magnetic resonance imaging (MRI). The day before implantation, RSCs were labeled with Feridex (a superparamagnetic iron oxide nanoparticles (SPIOs) contrast agent approved by FDA). Imaging was obtained by gradient reflection echo at 24 hours and every 10 days following transplantation for 3 months. We observed a susceptibility change with powerful signal damping on T2-weighted MRI. The RSCs were visible as dark tissue areas on the first day after implantation. The hypointense signal along the optic nerve intensified during 40 days. Within 6-month follow-ups, the patients were investigated by in vision,visual field,flash-visual evoked potentials (F-VEP) and functional MRI (fMRI) with light stimulation for functional recovery. The activation in fMRI maps was seen in the visual cortex. F-VEPs were improved with extended latency during six-month follow-ups. We have conducted our clinical study with considerable care, and taken every effort to gain as much information post-implant as possible. This pilot clinical study demonstrates that RSC engraftment and tracking after implantation are feasible, and allow further development of stem cell-based approaches to optic nerve regenerative medicine.

Poster Board Number: F-1007

CELL TRANSPORT SYSTEM FOR CLINICAL TRIAL USING CULTURED HUMAN ORAL MUCOSAL EPITHELIAL CELL SHEETS

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Purpose: To develop cell transport system for clinical trial using cultured human oral mucosal epithelial cell sheets. Material and Methods: We developed a cell transport container that can maintain and monitor the inside temperature and air pressure. Human oral mucosal epithelial cells obtained from two healthy volunteers were cultured on temperature-responsive culture dishes. Epithelial cell sheets were transported between Osaka University and Tohoku University using the cell transport container by airplane. The cell sheets were evaluated before and 12 hours after transportation. Histological and immunohistochemical analyses were performed. Cell viability and cell purity were evaluated by flow cytometry. Results:Temperature inside the container was kept above 32°C, and the change of air pressure was within 10 hPa during transportation. Cell sheets were well stratified and harvested successfully after transport. The expression patterns of keratin 3/76, p63, ZO-1 and MUC16 were equivalent before and after transport. The cell viability was 72.0% before transport and 77.3% after transport. Epithelial purity was 94.6% before transport and 87.9% after transport. Conclusion: Cultured human oral mucosal epithelial cell sheets can be safely transported with this new transport system.

Poster Board Number: F-1008

ISOLATION AND CHARACTERIZATION OF STEM CELLS FROM THE TRABECULAR MESHWORK OF THE HUMAN EYE

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Development of primary open-angle glaucoma (POAG) is associated with increased resistance to aqueous humour outflow through the dysfunctional trabecular meshwork (TM). The positive correlation of POAG manifestation with age and significant decrease in the TM cell population in glaucomatous TM, raises the possibility that it may be a stem cell-related disease. Putative stem cells are believed to exist in the TM. The purpose of the study is to isolate and propagate progenitors of the TM which could have the capacity to differentiate into functional TM cells to treat POAG. The TM-derived cells (TM-DC) express TM markers, and certain stem cell markers. Morphologically, they resemble mesenchymal stem cell (MSC). Gene expression and FACS analyses show that TM-DC express the positive MSC marker and lack the negative MSC markers. The capacity of TM-DC to differentiate into mesenchymal lineages of adipocytes, chondroblasts and osteoblasts, is indicative of their multipotency, another defining characteristic of MSC. Genomic characterization showed the TM-DC to have gene expression patterns similar to MSC derived from other tissues. These cells may have the potential to help replace lost or dysfunctional cells in the TM tissue associated with POAG.

Poster Board Number: F-1009

BMI1 AS A MODULATOR OF THE REGENERATIVE POTENTIAL OF MÜLLER CELLS IN THE RD1 MOUSE RETINA

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In lower vertebrates, Müller glia respond to retinal injury through dedifferentiation and regeneration of all major retinal cell types, including photoreceptors. In mammals, Müller cells retain some neuronal stem cell properties but fail to replace dead photoreceptors in the injured retina. In the Rd1 mouse model of retinitis pigmentosa, a mutation in the Pde6ß gene leads to a rapid retinal degeneration, inducing the activation of Müller cells (reactive gliosis) in which the neurogenic factor Pax6 is upregulated. During brain development, the neurogenic fate of progenitor cells is repressed by polycomb group (PcG) complex, thus inducing the transition to the astrogenic phase. The present study evaluates the role of Bmi1, a PcG protein, as a potent regulator of Müller cell-dependent regeneration. In Rd1 mice, only a single layer of photoreceptor nuclei is still present at P20. In the age-matched Rd1;Bmi1-/- retina, the thickness of the photoreceptor layer (ONL) corresponds to 75% of the wild-type ONL thickness. Since a similar level of TUNEL staining was detected in Rd1 and Rd1;Bmi1-/- photoreceptors at the onset of retinal degeneration at P12, the loss of Bmi1 may participate to a process that overcomes the initial loss of photoreceptors. We demonstrate here that Bmi1 loss in the Rd1 retina enhances the activation of Müller glia by downregulating p27Kip1, that these cells migrate to the ONL, and that several cells express the retinal progenitor mark-

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er Pax6 at the inner part of the ONL. These events are also initiated, but to a lesser extent, in the Rd1 retina. At P12, EdU incorporation assays show proliferating cells with atypical elongated nuclei at the inner border of the Rd1;Bmi1-/- ONL. Using the GFAP-Cre mouse (JAX), we are currently establishing lineage tracing experiments targeting Müller cells to determine the implication of this cell population in the maintenance of the Rd1;Bmi1-/- ONL thickness, and whether downregulation of Bmi1 may enhance their regenerative potential. In conclusion, our results show that the loss of Bmi1 efficiently reduces retinal degeneration in Rd1 mice and suggest that Bmi1 may act as a blockade to the regeneration of retinal cells by Müller glia in mammals.

Poster Board Number: F-1010

GENETIC DIFFERENCE IN THE PROLIFERATION OF MULLER GLIA AFTER RETINAL DAMAGE IN ADULT MOUSE

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Unlike the neural stem cell niche in the brain, neurogenesis is not normally detected in adult mammalian retina. On the other hand, when retinal neurons are damaged, retinal Muller glia turns into proliferative state and can differentiate into several types of retinal neurons in zebrafish, chick, and mammals. Compared to zebrafish, mammals have smaller numberof proliferative Muller glia after retinal damage, and limited types of retinal neurons are regenerated. Recent studies have shown that the addition of growth factors after retinal damage increased the proliferative Muller glia in mammalian retina. However, the addition of these factors alone did not cause Muller glia to proliferate in the intact retina, suggesting that other signals from degenerative environment are also important for the Muller glia to shift from quiescent to proliferative state. In order to access this point, we focused on the difference in proliferative potential of Muller glia, as well as in the changes of gene expression pattern during retinal degeneration between B6 and 129 mouse strains. After retinal damage, Muller glia expressed retinal progenitor markers in both mouse strains, but Ki67-positive proliferative Muller glia was detected only in the 129. To find the molecular mechanisms that contributed to this difference, we compared the retinal gene expression after damage between B6 and 129. We found several genes that were differentially expressed in 129 and B6 retina only after retinal damage. Among them, Hmga2, a chromatin-remodeling factor that is highly expressed in young neural stem cells, was highly expressed in 129. We also found that interferon-initiated genes were relatively high in 129 after retinal damage, and activation of innate immune system enhanced the proliferation of Muller glia. Our findings imply that in response to the retinal damage, some intrinsic signal including the innate immune system in surrounding cells may trigger the Muller glia to enter into cell cycle in the adult mouse retina.

Poster Board Number: F-1011

INCREASING RAT STEM CELL POPULATIONS IN CORNEA BY DRUG TREATMENT

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The cornea, a highly organized mass of proteins and tissue comprised of 5 layers: the epithelium, Bowman's layer, substantia propria (stroma), Descemet's Membrane, and the endothelium.The epithelium acts as a dynamic protective barrier from fluid, bacteria and other dangerous materials, and consists of squamous cells, suprabasal and inner columnar basal cells. Minor injuries to the eye can be repaired as limbal epithelial stem cells (LESCs), residing in the basal layer of the epithelium, divide asymmetrically across the basal layer of the epithelium as fully differentiated squamous cells are shed from the outer cornea. In some cases, such as extreme injuries involving chemicals or deep scratches, or diseases like Fuchs' Dystrophy or Keratoconus, naturally occurring epithelial replacement may not be enough, resulting in deterioration and visual impairment. Current treatments for these types of serious conditions involve corneal or stem cell transplantation. Corneal transplantations are fairly common, and have been known to be very successful in some cases; however, immune rejection occurs in about 20% of procedures. Stem cell transplantation procedures overcome this pitfall by using the patient's own corneal tissue from the non-effected eye, thus eliminating the chance for tissue graft rejection. Nonetheless, this type of procedure is limited in its use since some diseases or injuries may effect both eyes. Additionally, there are always risk factors associated with any invasive procedure. In order to overcome these difficulties, a non-invasive, small molecular pharmaceutical compound was developed. In our previous studies, this compound increased endogenous adult stem cells. In the current study, we formulated this compound into an eye drop, which was applied to the eyes of rats. The animals received BrdU (100mg/kg, i.p.) at the same time of treatment for detection of proliferating cells. The animals were sacrificed 2 weeks after treatment and the eyes were extracted. Serial saggital sections were made for immunohistochemical analysis for BrdU, and nuclei were counter stained with DAPI. The population of cells having BrdU positive nuclei were dose dependently increased by the treatment. These results suggest that the application of eye drops containing our compound increased proliferation of the endogenous corneal stem cell population, indicating the potential of this compound as a future regenerative therapy pharmaceutical for ocular disorders.

Poster Board Number: F-1012

CYTOCOMPATIBILITY OF COLLAGEN SCAFFOLD FOR RETINAL PIGMENT EPITHELIAL CELLS *IN VITRO*

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PURPOSE: Disease in retinal pigment epithelium (RPE) is a leading cause of human blindness, for which RPE cell replacement is a promising approach. This study is aimed to generate a RPE cell sheet on a biomaterial scaffold as a new transplant source. METHODS: RPE cells (ARPE-19) and "human iPS cell"derived RPE cells were seeded on a 100μm-thickness collagen membrane at a density of 10,000 cell/cm2.MTT assay was carried out to test the growth and viability of RPE cells on the scaffold. RT-PCR was used to check the gene expression of the RPE cells. Scanning electron microscope (SEM) and immunocytochemistry were performed to test the cell morphology,function and proliferation. RESULTS: RPE cells proliferated well *in vitro*. The cell number increased with the culture time on collagen scaffold as well as tissue culture plate (TCP). However, the rate of cell growth on scaffold was higher than that on TCP ($p < 0.05$). RT-PCR confirmed the specific expression of RPE65 and MERTK genes. Immunocytochemisty results showed expression of tight junction marker ZO-1 and E-cadherin. SEM displayed RPE cell

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characteristics. CONCLUSIONS: RPE cells grew and proliferated well on the collagen scaffold and maintained cell biological characteristics after a long-term culture on the collagen scaffold. In conclusion, the RPE cell sheet on the collagen membrane showed us a good cytocompatibility *in vitro* and would be a new approach of RPE cell transplantation.

Poster Board Number: F-1013

AN ANALYSIS OF THE MECHANISMS OF PHOTORECEPTOR CELL DEGRADATION IN A RETINITIS PIGMENTOSA PATIENT USING IPS CELLS

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It is very useful to analyze mechanisms of neurodegenerative diseases using iPS cells established from patients. Comparisons of the iPS cells established from the patients, who have mutations in the identical gene, have given us lots of knowledge. In the ophthalmology field, an analysis of a disease called retinitis pigmentosa (RP), in which rod cells in retina are gradually degenerated, using iPS cell has been reported. Some lines of iPS cells were established from the patients with mutations in different alleles and it was probed that they had different characters, such as sensitivity to the drugs. However, it cannot be denied that the difference between the iPS cell lines came from the other genetic background. Thus, we corrected a mutation of iPS cells established from an RP patient, which was considered to be the course of the disease, and compared with those with no gene therapy. The gene therapy was performed by knocking-in the wild type gene into the mutant allele. It is known that the rate of knocking-in a gene into human ES/iPS cells is much less than into mouse ES/iPS cells. So, we introduced a targeting vector using helper-dependent adenovirus, a recent technology for gene introduction with higher rate, and got gene treated iPS cells. Now, we are analyzing original and gene treated iPS cells from an RP patient, and trying to find a new therapy for RP by using these cells.

Poster Board Number: F-1014

DIFFERENTIATION OF INDUCED PLURIPOTENT STEM CELL-DERIVED NEURAL CREST CELLS INTO CORNEAL KERATOCYTES *IN VIVO*

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The usual procedure for treating corneal stromal disorders such as Avellino corneal dystrophy is transplantation of donor corneas. However, regenerative medicine using donor-derived cells and tissues, or ES cells include problems such as immunological rejection or ethical objection. Application of induced pluripotent stem cells (IPSCs) to regenerative medicine is expected to bypass these problems. Corneal stromal cells, or keratocytes, originate from cranial neural crest cells. In addition, we previously isolated Cornea-derived Precursors (COPs), which have characteristics of multipotent neural crest-derived stem cells, from the mouse corneal stroma. On the basis of these findings, we first tried differentiation of human IPSCs into neural crest cells (NCCs), to apply IPS cells to corneal stromal disorders. For efficient differentiation of IPSCs into NCCs, we examined modification of NSB method (Lee et al, 2010). By induction with modified-NSB method, over 30% of cells were isolated as NCCs using FACS. A problem to apply regenerative medicine to the corneal stroma lies in the difficulty of reconstructing the 3 dimensional corneal stroma. Therefore, we next tried direct injection of IPSCs-derived NCCs (IPS-NCCs) into the stroma. The IPS-NCCs labeled with fluorescent dye PKH26 were injected into mouse corneal stroma and examined for differentiation into keratocytes. Four weeks after injection, engraftment of the injected cells was observed by confocal fluorescence microscopy. Phalloidin staining revealed dendritic morphology, which is one of the characteristics of keratocytes. Furthermore, we found the expression of Keratocan, a corneal stroma specific keratan sulfate proteoglycan, in the engrafted cells. These results suggested that the IPS-NCCs is useful cell source for regenerative medicine of corneal stroma by cell transplantation.

Neural Cells

Poster Board Number: F-1015

ASTROCYTE-LIKE CELLS DERIVED FROM HUMAN ORAL MUCOSA STEM CELLS AMELIORATES A SCIATIC NERVE INJURY MODEL.

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Background: Sciatic nerve injury is a common pathology resulting in degeneration of distal axons and muscle denervation. Previous studies showed that direct administration of neurotrophic factors (NTFs) or via-differentiated stem cells with NTF overproduction (astrocytes-like cells), preserved and protected damaged motor neuron in the injured sciatic nerve. Recently, a unique stem cell population was isolated from the neural crest-derived lamina propria of the adult human oral mucosa, named hOMSC. hOMSC express constitutively markers of embryonic (Oct4, Sox2, Nanog), mesenchymal (CD73, CD90, CD105) and neural (nestin, snail and p75) stem cells which can be differentiated into cell lineages of the three germ layers. These hOMSC properties open a wide spectrum of therapeutic interventions, in which harvesting complexities and the ethical implications associated with embryonic or fetal stem cells can be avoided. Moreover, small biopsies of 2mm2 obtained by non-invasive procedures is required in order to gain access to a fully potent stem cells reservoir. Our objective was to evaluate hOMSC capabilities of becoming NTF secreting cells *in vitro* and then assess differentiated cell transplantation effectiveness in improving a rat model of sciatic nerve injury. Results: As a novel approach, we successfully converted hOMSC into astrocyte-like cells which effectively produce and secret NTFs. The obtained cells show typical astrocyte morphology and express characteristic astrocyte markers such as GFAP, S100β and the glutamate transporter GLT-1 (EAAT2). Moreover, these cells expressed GDNF, IGF-1 and high levels of BDNF and VEGF. Affected rats were transplanted with hOMSC-derived NTF secreting cells into the lesion site and motor behavior was assessed. Notably, rats injected with hOMSC-NTF showed improved motor function in contrast to saline or undifferentiated cells treated rats. This motor improvement can be significantly seen during the acute phase of recovery, following nerve crush and cell transplantation. Conclusions: Our findings show for the first time that astrocyte-like cells with enhanced NTF production can be successfully obtained through hOMSC differentiation. Moreover, these cells alleviate motor dysfunctions associated with acute phase recovery of sciatic nerve injury. Considering the many advantages of

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using oral mucosa stem cells and the results showed here, hOMSC emerge as a novel stem cell population for the autologous treatment of peripheral nerve injuries.

Poster Board Number: F-1016

STEM CELLS ISOLATED FROM HUMAN ORAL MUCOSA: A NOVEL TREATMENT FOR PARKINSON'S DISEASE

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Background: The oral mucosa possesses a high regenerative capacity regardless of the individual's age. Recently it was reported by our research group, a novel and unique stem cell population isolated from the neural crest-derived lamina propria of the adult human oral mucosa, named hOMSC. These cells express constitutively markers of embryonic (Oct4, Sox2, Nanog), mesenchymal (CD73, CD90, CD105) and neural (nestin, snail and p75) stem cells and differentiate into cell lineages of the three germ layers. These hOMSC properties open a wide spectrum of therapeutic interventions, in which harvesting complexities and the ethical implications associated with embryonic or fetal stem cells can be avoided. Moreover, small biopsies of 2mm2 obtained by non-invasive procedures is required in order to gain access to a fully potent stem cells reservoir. In this work we describe the potential application of an autologous cell replacement therapy for Parkinson's disease (PD). In this sense, we assessed the propensity of hOMSC to differentiate into dopaminergic (DA) neurons *in vitro* and evaluated the functional outcome of transplanted hOMSC derived-DA-like neurons, using the 6-OHDA PD rat model. Results: We demonstrated that hOMSC constitutively express not only a repertoire of neuronal genes but specific dopaminergic neurons markers such as tyrosine hydroxylase, Lmx1A and Nurr1. Exposure of hOMSC to a dopaminergic differentiation medium, induced a strong neuronal-like morphology in the vast majority of the cells, accompanied by a downregulation of early stem cell markers and upregulation of dopaminergic developmental transcription factors Nurr1, Pitx3, FoxA2, Otx2 and Lmx1A. Following dopaminergic differentiation, a significant increase in the regulated dopamine secretion was observed by HPLC. Finally, transplantation of DA-induced cells into the striatum of hemi-Parkinsonian rats, resulted in improvement of their behavioral deficits, as determined by apomorphine-induced rotational behavior. Conclusions: In this work we show for the first time the excellent propensity of hOMSC to differentiate into functional DA neurons *in vitro*, and to provide symptomatic relief in a PD rat model. Considering the many advantages of using oral mucosa stem cells and the promising results obtained here, hOMSC emerge as a novel stem cell population for autologous cell replacement therapy for Parkinson's disease.

Poster Board Number: F-1017

NEURONAL DIFFERENTIATION OF MOUSE HIPPOCAMPAL NEURAL STEM CELLS IS ENHANCED BY ELECTROMAGNETIC FIELD STIMULATION

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We recently reported that exposure of C57/BL6 mice to extremely low-frequency (50 Hz) electromagnetic fields (ELFEFs) stimulates adult hippocampal neurogenesis. The enhanced proliferation of neural stem/precursor cells (NSCs) residing in the subgranular zone of the dentate gyrus was associated with their increased neuronal differentiation followed by migration and functional integration of newborn neurons within the granular cell layer. To gain further insights into the ELFEFs' effects on hippocampal neurogenesis and identify the underlying molecular mechanisms we extended our studies to an in vitro model of NSCs isolated from the hippocampus of newborn C57/BL6 mice (P0). Undifferentiated NSCs were cultured in NeurobasalA/B27 medium containing the growth factors FGFb, EGF and PDGFbb. Under these culture conditions all NSCs exhibited immunoreactivity for nestin and they actively proliferated forming neurospheres. The NSC differentiation was induced by culturing cells dissociated from neurospheres in a medium in which FGFb, EGF and PDGFbb was replaced with 1% fetal calf serum. Our studies were carried out on undifferentiated NSCs (referred to as D0) and after 1-10 days' culture in the differentiation medium (time-points D1 to D10). At D0, 6-hour exposure to ELFEFs (50 Hz, 1 mT) significantly increased the proliferation of undifferentiated NSCs that was assessed by 5-bromo-2'-deoxyuridine (BrdU) incorporation. The percentage of BrdU-positive NSCs raised from 37±4 to 57±2% of total cells ($P<0.005$). The neuronal differentiation of NSCs was similarly enhanced by ELFEFs that increased the percentage of NSCs immunoreactive for the neuronal marker MAP-2 from 17±1% to 27±3% ($P<0.001$) at D6, and from 23±3% to 33±3% at D10 ($P<0.05$). When cells were cultured in the presence of the L-type voltage-gated Ca2+ (Cav1) channel blocker nifedipine (5 μM), the neuronal differentiation of NSCs was significantly lowered ($P<0.001$). Indeed, the percentages of MAP-2-positive cells were only 11±2% at D6 and 16±1% at D10. The blockade of Cav1 channels also prevented the ELEFEF-induced increases in neuronal differentiation that dropped from +60% to +1% at D6 and from +40% to +14% at D10. Quantitative RT-PCR analysis of NSC extracts revealed ELFEF-induced modulation in the transcription levels of the neuronal determination gene Mash1 and the anti-neuronal phenotype gene Hes1 at D0 (+20% and -17%, respectively vs. sham-exposed NSCs). At D1, only the differentiation genes Neurogenin1 and NeuroD1 were affected by ELFEF exposure. Indeed, their mRNA expression levels were increased by 28% and 48%, respectively, vs. sham-exposed NSCs. The ELFEF-induced enhancements of Neurogenin1 and NeuroD1 mRNAs were prevented by the presence of 5 μM nifedipine in the culture medium of NSCs. As a functional correlate of ELFEF-stimulated neurogenesis, C57/BL6 mice exposed to ELFEFs (7 h/day for 7 days) displayed memory enhancement as revealed by Morris water maze probe test performed 8 days after learning. Collectively, our findings suggest that electromagnetic fields are a useful tool to stimulate adult hippocampal neurogenesis *in vivo* and increase the neuronal yield of NSC differentiation in vitro. These effects are due to the ELFEFs' ability to modulate the expression of genes that are critical to NSC proliferation and dif-

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ferentiation and are, at least partly, dependent on intracellular Ca2+ signals generated by Cav1 channels.

Poster Board Number: F-1018

POSSIBLE ROLE OF IMPAIRED HIPPOCAMPAL NEUROGENESIS IN THE NEURODEGENERATION-LIKE ALTERATIONS INDUCED BY HERPES SIMPLEX TYPE-1 (HSV-1) IN RODENTS

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Several lines of evidence suggest that altered neurogenesis may contribute to the pathogenesis of neurodegenerative diseases. Recent studies from our and other groups suggested that chronic HSV-1 infection spreading to the central nervous system is a risk factor for Alzheimer's diseases. In particular, we found that HSV-1 increased the neuronal excitability of rat cortical neurons thus triggering Ca^{2+}-mediated phosphorylation of amyloid precursor protein (APP) and its processing. As a consequence, HSV-1 caused intracellular and extracellular accumulation of amyloid-β protein (Aβ) and other APP fragments (APPFs) with documented neurotoxic activity. In the present study we investigated the effects of both HSV-1 and the HSV-1-induced products of APP processing on neural stem cells (NSCs) isolated from the hippocampus of newborn (P0) C57BL/6 mice. Undifferentiated (nestin-positive) NSCs were cultured in NeurobasalA/B27 medium containing the growth factors FGFb, EGF and PDGFbb. The NSC differentiation was obtained by culturing cells dissociated from neurospheres in a medium in which the growth factors was replaced with 1% fetal calf serum. NSCs were studied under proliferating conditions (referred to as time point D0) and after 1-10 days' culture in the differentiation medium (referred to as time-points D1-D10). Under the latter experimental conditions, the percentages of cells displaying immunoreactivity for the neuronal marker MAP-2 were 11±1% of total cells at D3, 17±1% at D6 and 23±3% at D10. NSCs were infected with HSV-1 (0.5 MOI) immediately before starting the differentiation protocol. At D3, virtually all NSCs were infected, as documented by their immunoreactivity for HSV-1, and the vast majority of HSV-1-positive NSCs also exhibited intracellular accumulation of Aβ42, that was only weakly labelled in few mock-infected cells. Incidentally, this finding also documented that the enzymes required for APP cleavage, i.e., β- and γ-secretases are fully active in early phases of NSC differentiation. To determine the impact of the HSV-1-induced Aβ42 accumulation on NSC biology we tested the effects of this protein on both undifferentiated and differentiating cells. Proliferation of undifferentiated NSCs (D0), investigated by 5-bromo-2'-deoxyuridine incorporation, was significantly inhibited by 24-h treatment with 200 nM Aβ42 (-24%; $P<0.05$). The same treatment also reduced the percentage of MAP-2-positive cells at D3 (-27%; $P<0.05$) and the mean numbers of viable cells within microscopic fields (-44% of control NSCs; $P<0.01$). Finally, the conditioned medium of HSV-1-infected SH-SY5Y human neuroblastoma cells containing Aβ42 and other APPFs exerted a powerful toxic effect on NSCs (> 90% cell death at D3). In conclusion, our findings suggest that in hippocampal NSCs HSV-1 causes intracellular accumulation of Aβ42 that significantly reduces viability, proliferation and differentiation of NSCs toward the neuronal phenotype. These data allow us to hypothesize that impaired hippocampal neurogenesis contributes with the previously documented functional and molecular alterations induced by HSV-1 in the brain to produce neuropathological conditions that are reminiscent of neurodegenerative diseases.

Poster Board Number: F-1019

SMALL MOLECULE SCREENING OF NEURAL DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS

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Human embryonic stem cells (hESCs) are a promising source of cells for regenerative medicine, as well as a tool for toxicity and drug development. These endeavours require pure populations of disease relevant cell types. Therefore, it is crucial to the field to develop efficient and reproducible differentiation protocols. Here, we demonstrate how medium-throughput screening with a suite of small molecules and growth factors can be utilized to produce a single step differentiation protocol. preliminary study of 27 compounds/growth factors in combinations of two, utilizing poly-L-lysine/fibronectin and Collagen I as extracellular matricies, and immunocytochemical markers, O1, O4, Nestin, Engrailed1, Lmx1a and others identified several conditions of interest. Most notably a five day differentiation protocol to produce a high yield of oligodendrocytes, as shown by the expression of O1/O4, was discovered. This could have a profound affect as a cell replacement model for oligodendrocyte affected diseases.

Poster Board Number: F-1020

GENERATION OF GAD65 NEURONS FROM DISEASE AFFECTED HUMAN EMBRYONIC STEM CELLS FOR HIGH-THROUGHPUT SCREENING

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Disease affected human embryonic stem cells (hESCs) can be a source of differentiated cells for advancing Huntington's disease pathology and for the identification and characterisation of potential therapeutics. We demonstrate the ability to reliably supply mixed populations of neurons, astrocytes, and oligodendrocytes from normal and pre-implantation genetic diagnosis (PGD) derived disease affected embryonic stem cell lines. Routine testing of neural markers show that hESCs are efficiently differentiated to neural cell types by a two-step differentiation protocol utilizing a suite of Genea Neural Media; cells were >60% MAP2+, 5% GFAP+, and >30% oligodendrocytes. In addition, neurons were >70% GAD65/67+, suggesting this method is an efficient source of GABAergic neurons for cell replacement or Huntington's disease related drug development studies. This is reproducible in multiple Huntington's disease affected stem cell lines. We possess a number of other PGD derived stem cell lines with neurological disorders. This includes our disease affected stem cell line from an Incontinentia Pigmenti affected embryo which may improve understanding of the role GABAergic neurons play in this disease. We have also shown that neurodifferentiated DIV30+ cells can be shipped live (on heat packs) in 96-well plates, or frozen. This allows a reliable supply of neurodifferentiated cells to be available worldwide for cell-based high-throughput drug screening, as well as for future clinical applications.

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Poster Board Number: F-1021

MODELLING GENE-ENVIRONMENT INTERACTION IN FETAL NEUROGENESIS AND SCHIZOPHRENIA: EFFECT OF DISC1 AND TNF-A ON HUMAN NEURAL PROGENITOR CELLS

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Gene-environmental interactions (GEI) are implicated in the pathogenesis of psychiatric disorders such as schizophrenia (SZ). Disrupted-in-Schizophrenia1 (DISC1) has been identified as a promising susceptibility gene, while prenatal exposure to pro-inflammatory cytokines (including tumour necrosis factor-alpha; TNF-α) has been implicated as an environmental risk factor for SZ. Purpose: This study aimed to generate an *in vitro* model of GEI using previously established human neural progenitor cells (hNPCs). Specifically, the effects of DISC1 loss-of-function (LOF) and TNF-α on hNPC biology were studied by investigating cell cycle and survival. Methods: Generation of DISC1 transgenic hNPCs has previously been reported. DISC1 transgenic or wild-type hNPCs were treated with vehicle or 50ng/ml TNF-α (TNF50). At day 7, gene expression was analysed by qPCR and hNPC counts were performed by trypan blue exclusion method. At days 3, 5 and 7, immunocytochemistry was performed for Ki-67 or active caspase-3. Immunofluorescent images were quantitated using Image J. Results: TNF50 increased the expression of TNF-α and its receptors and reduced hNPC numbers. Moreover, there was an apparent combined effect of TNF50 and DISC1 LOF on reduced hNPC number. Immunocytochemistry revealed that TNF50 had a predominant effect on cell cycle with a marked reduction in total Ki67 staining corresponding to G2/M phase hNPCs, and increased numbers of apoptotic hNPCs with active caspase-3 expression. Interestingly, DISC1 LOF potentiates TNF50 induced increase in numbers of active caspase-3 positive hNPCs. Further studies are underway to determin molecular signalling events leading to cell death. Conclusion: DISC1 LOF augments the effect of TNF-α on molecular and morphological indicators of programmed cell death. Our *in vitro* modelling demonstrates the interaction between a genetic risk factor and exogenous stressor on hNPC biology relevant to human neurodevelopment and neuropsychiatric diseases including SZ.

Poster Board Number: F-1022

PRE-DIFFERENTIATION OF SUBVENTRICULAR ZONE DERIVED HUMAN NEURAL PROGENITOR CELLS ENHANCES NEURONAL REPOPULATION FOLLOWING TRANSPLANTATION WITHIN THE STROKE DAMAGED BRAIN

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There is a compelling need to develop effective treatment strategies for patients affected by stroke. The use of stem cell based therapies to promote brain repair may offer new hope for improving patient recovery after stroke. To this end, we have isolated and characterized human neural progenitor cells (hNPCs) derived from subventricular zone (SVZ) that can be differentiated into neural cells including GABAergic neurons. Purpose: To investigate the effect (histological and behavioural outcomes) of undifferentiated and pre-differentiated SVZ-hNPC transplants into the rat brain 7 days post-stroke. Methods: The middle cerebral artery was constricted by endothelin-1 (ET-1) in conscious rats; group 1 receiving undifferentiated SVZ-hNPCs, group 2 receiving pre-differentiated SVZ-hNPCs and group 3 receiving vehicle with media alone. Neurological outcome was assessed by neurological deficit score, sticky label test, and cylinder test. 7 days after ET-1 stroke undifferentiated, pre-differentiated SVZ-hNPC's or vehicle were stereotaxically injected into the rat brain at 8 predetermined sites to target both the striatum and cortex. Brains were harvested 28 days post-transplant. SVZ-hNPC survival, proliferation and neurite outgrowth were assessed using immunohistochemistry and confocal microscopy. Results: Neurological functional assessment revealed spontaneous recovery across all three treatment groups. Undifferentiated as well as pre-differentiated SVZ-hNPCs survived the grafting procedures and within the stroked affected brain. Immunohistochemical analysis revealed pre-differentiated SVZ-hNPCs maintained their neuronal phenotype post-transplant as evidenced by human nuclear antigen (hNA) colabeled with β-III-tubulin and GABA. Among the implanted undifferentiated SVZ-hNPCs, a small percentage was immunostained for hNA and β-III-tubulin while the majority of cells were double-positive for hNA and glial fibrillary acidic protein indicating differentiation into astrocytes. Conclusion: Pre-differentiating SVZ-hNPCs into neuronal cells prior to transplantation results in a greater number of SVZ-hNPC-derived neuronal populations within the damaged region of the stroke affected brain. Our findings may shed a light in use of SVZ-hNPCs to promote restoration of the stroke affected brain.

Poster Board Number: F-1023

HUMAN PARTHENOGENETIC STEM CELL DERIVED A9 DOPAMINERGIC NEURONS TO TREAT PARKINSON'S DISEASE

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Human parthenogenetic stem cells (hpSCs) have recently been proposed as an alternative source for cell replacement therapies. hpSC's main advantage over other pluripotent stem cells is the homozygosity at the HLA loci, which significantly reduces immunogenicity and simplifies immune matching in clinical applications. Another important advantage of hpSCs, is that their derivation does not involve the destruction of a viable human embryo. hpSCs are derived from unfertilized oocytes that have been chemically activated to develop into a parthenogenetic blastocyst. hpSC lines are isolated from the inner cell mass of the parthenogenetic blastocyst. hpSCs behave similarly to human embryonic stem cells (hESC) in proliferative and multilineage differentiation potential. The parthenogenetic derived neural stem cells (NSCs) express early progenitor markers, such as Nestin, Sox1, Sox2, Musashi-1, Pax6 and have the capacity to differentiate into various neuronal subtypes. The aim of this study is to derive a stable population of A9 dopaminergic neurons from hpSCs and further establish in vitro and *in vivo* functionality. Using immunocytochemistry and RT-PCR, we found that the differentiated parthenogenetic derived neurons exhibit a midbrain phenotype and express such markers as TH, GIRK2, PITX3, NURR1, LMXA1, EN1 and do not express CALB. Using HPLC and ELISA, we monitored the neurons production of Dopamine. We determined that hpSC derived dopaminergic neurons are capable of firing action potentials by whole cell electrophysiology. We also

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show that hpSC derived neuronal cells are capable of surviving *in vivo*. We have begun *in vivo* functionality studies of parthenogenetic derived A9 dopaminergic neurons in Parkinsonian rat models. We are currently conducting behavioral tests such as the cylinder test and amphetamine and apomorphin induced rotations. Our Initial studies indicate that hpSC derived A9 dopaminergic neurons can be an excellent source of neurons for the treatment of Parkinson's disease.

Poster Board Number: F-1024

INVESTIGATIONS INTO THE TREATMENT OF STROKE WITH HUMAN PARTHENOGENETIC STEM CELL DERIVED NEURAL PRECURSORS USING VARIOUS DISEASE MODELS

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The chemical stimulation of unfertilized oocytes generates parthenogenetic blastocysts from which human parthenogenetic stem cells (hpSCs) are derived. HpSCs have very similar properties to human embryonic stem cells (hESCs), they proliferate indefinitely and have multilineage differentiation capacity. However hpSCs do not raise the same ethical concerns as hESCs, because their generation does not involve the destruction of a viable human embryo. hpSCs also have a very important clinical advantage over hESCs that is they are homozygous at the HLA loci, which potentially reduces immunogenicity and simplifies immune matching when considering implantation. hpSC may also be useful for the generation of neural precursors for the treatment of Stroke. Using established protocols, the spontaneous differentiation of hpSC lines in neural induction media gave rise to neural rosettes which were mechanically isolated and expanded to obtain neural precursor cells. These cells were maintained in medium containing bFGF and EGF and were extensively characterized by PCR, FACS, and immunocytochemistry. Expression of markers such as PAX6, SOX1, NES, and MSI-1 confirmed the identity of the cells. These neural precursor cells are stable to passaging and cryopreservation and have been shown to have the ability to further differentiate in vitro into functional neurons. Future work will focus on the application of hpSC derived neural precursors with *in vivo* models of Stroke, such as the acute arterial occlusion model. hpSC could potentially represent an important and immunologically superior source of neural precursor cells for cell therapy of Stroke.

Poster Board Number: F-1025

NEURAL GENE EXPRESSION AND MIRNA PROFILING IN HUMAN NEURAL STEM CELLS (HNSCS) FOLLOWING DIFFERENTIATION ON A THREE DIMENSIONAL SCAFFOLD

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Much attention is being devoted to the potential uses of stem cells in human biology and medicine. However, our understanding of how to control and exploit their full developmental potential is currently limited. Conventionally in vitro hNSC differentiation is conducted on two dimensional (2D) substrates, which is far removed from the environment which cells experience in living tissues. *In vivo* cells are in a three dimensional (3D) structure that is optimal for their growth and function. The CTX0E03 cell line is a hNSC line manufactured to Good Manufacture Procedure (GMP) and currently in a Phase I clinical trial for chronic stroke disability in Scotland. Here we have characterized the ability of CTX0E03 hNSCs to differentiate on commercially available 3D scaffolds (Alvetex, amsbio, UK). Neurite growth was significantly enhanced when CTX0E03 cells were grown in a 3D environment (195.76 µm +/- 9.36) compared to traditional flat surfaces (59.33µm +/- 7.00), resulting in more mature neuronal and glial phenotypes. We performed realtime PCR analysis to determine the expression patterns of neuronal (βIII tubulin, DARPP32, MAP2, and DCX), and glial (GFAP, S100, and GALC) genes. All these genes, with the exception of S100, were found to be significantly increased in 3D grown samples compared to standard 2D ones. Furthermore we investigated if microRNAs (miRNAs) could be involved in RNA control of stem cell behaviour on 3D. Using a Cell development and Differentiation miRNA PCR array (Sabiosciences, Qiagen, UK), we have identified a set of miRNAs that are significantly up- and down-regulated in 3D cultured CTX0E03, compared to 2D standard differentiation protocol. Significant change was defined as +/- 1.5 fold up and down regulation. Among the up-regulated miRNAs we found: hsa-let-7i, hsa-let-7c, hsa-let-7b, hsa-miR-125b, hsa-miRNA-9, has-miR-26a and has-miR-181a, known to prominently up-regulate neurogenesis. Among the down-regulated miRNAs we found hsa-miR-302, and has-miR-18a, (also down-regulated in an in vitro model of neurogenesis), has-miR-17, has-miR-20a (involved in monocytopoiesis), has-miR-23b (related with genes correlated with neurogenesis) and has-miR-21 (involved in stemness through regulation of self-renewal and pluripotency). HNSCs grown on a 3D tissue-like environment showed enhanced expression of neural/glial genes and regulation of miRNAs, demonstrating the validity of the hNSC 3D differentiation assay for studying RNA/miRNA regulation in neural development.

Poster Board Number: F-1026

NEURAL GENE EXPRESSION AND MIRNA PROFILING IN HUMAN NEURAL STEM CELLS (HNSC) FOLLOWING DIFFERENTIATION ON A THREE DIMENSIONAL SCAFFOLD

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Much attention is being devoted to the potential uses of stem cells in human biology and medicine. However, our understanding of how to control and exploit their full developmental potential is currently limited. Conventionally in vitro hNSC differentiation is conducted on two dimensional (2D) substrates, which is far removed from the environment which cells experience in living tissues. *In vivo* cells are in a three dimensional (3D) structure that is optimal for their growth and function. The CTX0E03 cell line is a hNSC line manufactured to Good Manufacture Procedure (GMP) and currently in a Phase I clinical trial for chronic stroke disability in Scotland (UK). Here we have characterized the ability of CTX0E03 hNSCs to differentiate on commercially available 3D scaffolds (Alvetex, amsbio, UK). Neurite growth was significantly enhanced when CTX0E03 cells were grown in a 3D environment (195.76 µm +/- 9.36) compared to traditional flat surfaces (59.33µm +/- 7.00), resulting in more mature neuronal and glial phenotypes. We performed real-time PCR analysis to determine the expression patterns of neuronal (βIII tubulin, DARPP32, MAP2, and DCX), and glial (GFAP, S100, and GALC) genes. All these genes, with the exception of S100, were found to be significantly increased in 3D grown samples compared to standard 2D ones. Furthermore we investigate if microRNAs (miRNAs) could be involved in RNA control of stem cell behaviour on 3D. Using a Cell development and Differentiation miRNA PCR array (Sabiosciences, Qiagen, UK), we have identified

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a set of miRNAs that are significantly up- and down-regulated in 3D cultured CTX0E03, compared to 2D standard differentiation protocol. Significant change was defined as +/- 1.5 fold up and down regulation calculated according to the ΔΔct method. Among the up-regulated miRNAs we found: hsa-let-7i, hsa-let-7c, hsa-let-7b, hsa-miR-125b, hsa-miRNA-9, has-miR-26a and has-miR-181a, known to prominently up-regulate neurogenesis. Among the down-regulated miRNAs we found hsa-miR-302, and has-miR-18a, (also down-regulated in an in vitro model of neurogenesis), has-miR-17, has-miR-20a (involved in monocytopoiesis), has-miR-23b (related with genes correlated with neurogenesis) and has-miR-21 (involved in stemness through regulation of self-renewal and pluripotency). HNSCs grown on a 3D tissue-like environment showed enhanced expression of neural/glial genes and regulation of miRNAs, demonstrating the validity of the hNSC 3D differentiation assay for studying RNA/miRNA regulation in neural development.

Poster Board Number: F-1027

INTRASTRIATAL HUMAN NEURAL STEM CELL TRANSPLANTATION ENHANCES NEUROGENESIS FROM ENDOGENOUS NEURAL STEM CELLS AND IMPROVES RECOVERY AFTER STROKE.

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[Objective]Neurogenesis from endogenous neural stem cells (NSCs) and NSC transplantation have been suggested as novel approaches to reconstruct stroke-damaged neural circuitry. In rats, endogenous NSCs in subventricular zone (SVZ) produce new neurons migrating into striatum during several months after stroke but their long-term survival is poor. For maximum recovery, enhancement of endogenous neurogenesis should probably be combined with NSC transplantation. NSCs have beneficial effects not only through cell replacement but also by trophic actions, neuroprotection, and modulation of inflammation. Our preliminary data suggested that transplanted human NSCs might enhance endogenous neurogenesis in intact brain. The aim of this study was to analyze the interaction between endogenous neurogenesis and human-derived NSC transplantation in stroke-damaged brain. [Material and Methods] T cell-deficient rats were subjected to unilateral 1-hour middle cerebral artery occlusion and human foetal NSCs or vehicle were transplanted into ipsilateral striatum 48 hours thereafter. Behavioural function was assessed using stepping and cylinder tests every 3 weeks. Animals received BrdU injections for 2 weeks at 4 weeks before termination, and were perfused 6 or 14 weeks after transplantation. Specimens were immunohistochemically assessed for endogenous neurogenesis, graft survival and inflammation after stroke. [Results] We observed higher numbers of Ki67+ proliferating cells in the SVZ of the human NSC grafted group than in the vehicle group both at 6 and 14 weeks after transplantation. Also the numbers of Dcx+ migrating neuroblasts and BrdU+/Fox3 (NeuN) + newly formed mature neurons in the ischemic striatum were significantly higher in the human NSC transplantation group compared to the vehicle group at both time points. Human NSC transplantation reduced the number of Iba1+/ED1+ macrophages in the ischemic striatum. Grafted human NSCs were observed in all rats, but the number and the morphology of the grafts varied in the group. Animals with human NSCs grafts showed functional recovery in stepping test at 6 weeks and thereafter, whereas vehicle-injected animals did not. In cylinder test, the NSC transplanted animals showed improved recovery of impaired forelimb use at 12 weeks. [Conclusion]Intrastriatal human NSC transplantation enhances the proliferation, migration and maturation of endogenous NSCs after stroke, and this effect is long-lasting. Transplantation of human NSCs also reduces striatal inflammation and ameliorates neurological deficits after stroke. Our findings indicate that human NSC transplantation promotes endogenous neurogenesis from SVZ and modulates inflammation and support the idea that combination of NSC transplantation and stimulation of endogenous neurogenesis may become of value for functional restoration after stroke.

Poster Board Number: F-1028

MUTUAL INTERACTION OF BIFUNCTIONAL MICRORNA-9/9* AND THE NOTCH SIGNALING PATHWAY IN HUMAN NEURAL STEM CELLS

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MicroRNAs (miRNAs) are small non-coding RNA molecules that regulate gene expression at a post-transcriptional level. In the brain they are expressed in a temporally and spatially controlled manner and contribute to different aspects of nervous system development. In particular, miRNAs have been shown to regulate neural stem cell differentiation. One of the best-studied miRNAs in the brain is the bifunctional miRNA-9/9*. It is known to modulate neural stem cell properties and is deregulated in several brain tumors. We assessed the impact of miR-9/9* on a homogeneous population of human embryonic stem cell-derived neuroepithelial-like stem cells recently derived in our lab (lt-NES). Our gain-of-function analyses indicate that both miR-9 and miR-9* contribute to the shift of lt-NES cells from self-renewal to neuronal differentiation. Following up on previous data showing that Notch signaling is essential for lt-NES cell maintenance, we used Notch gain- and loss-of-function studies to explore potential interactions between this pathway and miR-9/9*. Here we show that Notch controls the expression of miR-9/9* at the transcriptional level. Using in *silico* promoter analyses we identified putative binding sites for RBPj, the transcriptional partner of Notch intracellular domain (NICD), upstream of all three genomic miRNA-9/9* loci. Chromatin immunoprecipitation confirmed direct binding of NICD/RBPj to these genomic sites. Using target prediction analyses we further identified putative binding sites for miR-9 and miR-9* in the 3'-UTR of several members of the Notch signaling pathway, which we are currently validating. Our data point to the existence of a feedback interaction between bifunctional miRNA-9/9* and the Notch pathway, which may contribute to controlling the delicate balance between self-renewal and differentiation of human neural stem cells.

Poster Board Number: F-1029

PARKINSON'S DISEASE MODELING WITH PATIENT-SPECIFIC INTEGRATION-FREE HUMAN INDUCED PLURIPOTENT STEM CELL LINES

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The motor symptoms of Parkinson's disease (PD) are mainly caused by the loss of midbrain dopaminergic neurons. The genetic studies in familial PD cases have identified mutations in several genes, including LRRK2, parkin, and α-synuclein. The advances in induced pluripotent stem cell (iPSC) technology enable the generation of iPSC lines from somatic cells of PD patients by reprogramming with defined factors. iPSC lines carrying PD-associated mutations can be differentiated into dopaminergic neurons and used for disease

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modeling, testing of the effect of various molecules on dopaminergic neurons, and as a source of cells for the cell replacement therapy. An issue in using iPSC technology for potential therapy and disease modeling is that iPSC are typically produced by transduction of somatic cells with integrating viruses that carry reprogramming factors; thus, this technique raises a risk of transgene reactivation and insertional mutagenesis. We focus on developing integration-free patient-specific iPSCs and differentiating them into dopaminergic neurons. We have successfully reprogrammed CD34-positive cells from healthy cord blood donors and skin fibroblasts from PD patients with LRRK2 and PARK2 mutations into iPSCs using two different non-integrating approaches. Generated iPSCs show human embryonic stem cell-like morphology and express pluripotency markers TRA1-60, NANOG, SOX2 and OCT4. We have characterized iPSCs derived from CD34-positive cells in greater detail and found that they have normal karyotypes, are vector-free, and can differentiate into all three germ layers. Importantly, we were able to derive neural stem cells (NSCs) from vector-free iPSCs and differentiate them into dopaminergic neurons, confirming the usefulness of this approach for generation of integration-free iPSCs, NSCs, and dopaminergic neurons. We are currently producing NSCs and dopmainergic neurons from PD patient-specific vector-free iPSCs, with a goal of using them for disease modeling and gene function studies.

Poster Board Number: F-1030

CONTRA-LATERALLY TRANSPLANTED HUMAN EMBRYONIC STEM CELL-DERIVED NEURAL PRECURSOR CELLS (ENSTEM-A) MIGRATE AND IMPROVE BRAIN FUNCTIONS IN STROKE-DAMAGED RATS

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Transplantation using neural precursor cells (NPCs) is known to be a promising approach to ameliorate behavioral deficits after stroke in a rodent model of middle cerebral artery occlusion (MCAo). Previous studies have shown that transplanted NPCs migrate toward infarct lesion, survive and differentiate into mature neurons to some extent. However, the spatio-temporal dynamics of NPC migration following transplantation into stroke animals have yet to be elucidated. In this study, we investigated the fates of human embryonic stem cell (hESC)-derived NPCs (ENStem-A) following transplantation into the contra-lateral side to the infarct region using 7.0T animal MRI for 8 weeks. T2 and T2*-weighted MRI analyses indicated that the migrating cells were clearly detectable at the infarct boundary zone by 1 week, and the intensity of MRI signals robustly increased within 4 weeks after transplantation. Afterwards, the signals were slightly increased or unchanged. At 8 weeks following transplantation, we carried out prussian blue staining and immunohistochemical staining using human-specific markers, and found that high percentages of transplanted cells migrated to the infarct boundary and they were mainly CXCR4-positive. We also observed that the migrating cells expressed markers for various stages of neural differentiation, including nestin, Tuj1, NeuN, TH, Darpp-32, SV38, indicating that the transplanted cells may partially contribute to the reconstruction of the damaged neural tissues after stroke. Interestingly, we found that the extent of gliosis (GFAP+ cells) or apoptosis (TUNEL+ cells) were significantly decreased in the cell transplanted group, suggesting positive roles of hESC-NPCs for reducing scar formation or cell death after stroke. No tumor was formed in this study. We also carried out various behavioral tests, including rotarod, stepping and mNSS tests, and found that the transplanted animals exhibited significant improvements in sensorimotor functions during 8 weeks following transplantation. Taken together, these results strongly suggest that hESC-NPCs have the capacity to migrate to the infarct region, form neural tissues efficiently, and contribute to the behavioral recovery. This work was supported by a grant of the Korea Healthcare technology R&D project, Ministry for Health, Welfare & Family Affairs, Republic of Korea (A111016).

Poster Board Number: F-1031

HUMAN NEURAL STEM CELL-MEDIATED TARGETING OF THERAPEUTIC NANOPARTICLES TO BRAIN TUMORS

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There is a critical need for tumor-selective therapeutic delivery strategies when treating primary brain tumors and brain metastases. In pre-clinical models, tumor-tropic neural stem cells (NSCs) can overcome distribution obstacles associated with even advanced nanoparticle (NP)-mediated drug delivery: NSCs home to invasive tumor foci and avoid off-target spleen and liver deposition, NSCs penetrate into hypoxic tumor regions, and NSCs traverse the blood-brain barrier (BBB) to access intracranial tumors. This property makes NSCs an exciting platform to improve the biodistribution of therapeutic nanoparticles used to treat brain malignancies. We previously established a clonal, human NSC line (HB1.F3.CD) that is currently being used in Phase I clinical trials to mediate enzyme/prodrug therapy in recurrent glioma patients. We recently demonstrated that HB1.F3.CD NSCs can be combined with endocytosed iron NPs for diagnostic purposes. Here we hypothesize that these clinically relevant NSCs can be combined with surface-conjugated NPs for therapeutic purposes. To make the NSC surface amenable for NP coupling, established methods were used to biotinylate surface sialic acid residues. Cells were then incubated with avidin to generate a stable bridge that can bind biotinylated NPs. FACS analysis, immunological staining, and avidin quantification assays show that 85 ± 9% of NSCs contain surface avidin moieties (~1 x 107 moieties/cell); and cell viability was unimpaired (98% viable). Avidinylated NSCs were incubated with two different therapeutic NP preparations: 1) drug-loaded polymeric NPs that release tumor-toxic drugs in response to the acidic tumor environment, and 2) gold (Au) NPs that generate tumor-toxic heat upon exposure to otherwise nonharmful radiofrequency radiation. Biotinylated AuNPs were obtained commercially, while the drug-loaded NPs (diameter = 150-300 nm) were fabricated from synthesized polymers (Biotin-PEG-polyDPAEMA) using an emulsion-evaporation approach. Particles were confirmed to contain surface-biotin moieties, and exhibit tight pH-dependant drug release between pH 6.5-7; (97 ± 2% retention at pH=7.4, 20 ± 10% retention at pH=6.5). After coupling NPs to the NSC surface, dark field, confocal, scanning and transmission electron microscopy were used to confirm the surface-localization of NPs. NSC-NP combinations retained unimpaired viability and migration to tumor conditioned media during in vitro Boyden-chamber chemotaxis assays. A maximum of 175 ± 23 surface-bound particles/cell were retained after migration. *In vivo* biodistribution studies demonstrate that NSC-NP combinations retain tumor tropism and can cross the BBB to access intracranial tu-

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mor foci, with no off-target deposition observed. In vitro and *in vivo* therapeutic efficacy studies with both NSC-NP combinations are now underway. So far, in vitro drug toxicity trials have confirmed pH-dependant tumor cell killing (Temozolomide and Docetaxel respectively killing U251 glioma and MDA-MB-231-BR metastatic breast cancers); meanwhile photothermal trials have confirmed that AuNP-NSCs exposed to long-wavelength electromagnetic radiation generates thermal energy sufficient to kill exposed cells. Together, this research combines human NSCs to achieve significant improvements in NP-delivery to brain tumors, which should translate to improved clinical outcomes and minimized side effects for patients with invasive or drug resistant brain tumors.

Poster Board Number: F-1032

PREFERENTIAL RETINOIC ACID-DEPENDENT DEMETHYLASE ACTIVITY OF UTX RATHER THAN JMJD3 ON THE CLUSTRED HOX GENES DURING NEURAL DIFFERENTIATION OF HUMAN EMBRONIC STEM CELLS

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Gene activation of *HOX* clusters is an early event in embryonic development. These genes are highly expressed and been active in the vertebrate nervous system. Based on the presence of Retinoic Acid Response Element (RARE) in regulatory region of many of *Hox* genes, it is deduced that Retinoic Acid (RA) can influence on epigenetic and consequently expression pattern of *HOX* during RA-induced differentiation of embryonic model systems. In this investigation the expression level as well as epigenetic regulation of *Hox* genes were analyzed in human embryonic stem cells (hESC) differentiated to neural precursor cells (NPC), in the presence and absence of Retinoic Acid. Expression analysis data significantly showed increased mRNA levels of all 1-5 *Hox* genes of the four A-D clusters in the presence of RA. Epigenetic analysis of the *Hox* gene regulatory regions also showed a significant decrease in methylation of histone H3K27 parallel to a preferential incorporation of the demethylase UTX rather than JMJD3 in RA-induced neural differentiated cells. This finding interestingly showed the functional role of UTX in epigenetic alteration of *HOX* clusters during RA-induced neural differentiation, the activity could not be detectable for the demethylase JMJD3, during this developmental process.

Poster Board Number: F-1033

A HUMAN PLURIPOTENT STEM CELL BASED MODEL FOR SPORADIC PARKINSON'S DISEASE

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Parkinson's disease (PD) is the second most common neurodegenerative disease affecting over one million people. Its characteristic pathology is degeneration of dopamine neurons in the substantia nigra pars compacta. The vast majority of parkinsonian cases (aprox. 90%) are sporadic. Epidemiological studies, as well as results obtained in animals and human tumor cell lines, have suggested that pesticides are involved in the pathogenesis of sporadic PD. However, a major hindrance in these studies is the absence of a relevant human model. We developed an effective protocol for the differentiation of human embryonic stem cells (hESC) and induced pluripotent stem cells (iPSC) through dopaminergic neurons. We tested commonly used pesticides: Rotenone, Paraquat and Maneb, in these cells. The three pesticides showed different neuronal toxicity. The loss of TH+ neurons and aggregation of alpha-synuclein observed are cardinal PD features. We propose the use of human pluripotent stem cell-derived dopaminergic cultures treated with pesticides as an *in vitro* human model for environmental origin of PD. This model will help further understanding of the ontogeny and molecular mechanisms underlying sporadic PD, and will be useful in the search of therapeutic targets.

Poster Board Number: F-1034

CD200 AND HLA-A,B,C ENABLES THE ISOLATION OF NEURONS FROM NEURAL INDUCTION CULTURES OF HUMAN EMBRYONIC STEM CELLS.

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The differentiation of human pluripotent stem cells to neural ectoderm presents an opportunity to study human neurogenesis and neurodegenerative diseases. Previous work has demonstrated that it is possible to identify distinct cell populations that represent developmental transition points in neural induction cultures using cell surface markers and fluorescence activated cell sorting (FACS). To identify additional cell surface signatures of neural cell types we combined intracellular marker expression with cell surface immunophenotyping. We performed a large unbiased screen of 242 antibodies that recognize cell surface epitopes while analyzing for intracellular expression of neural stem cell markers Pax6, Sox1 and Sox2 and the neuronal marker doublecortin (DCX). A comparative analysis of cell populations defined by the expression of Pax6, Sox1 and DCX revealed that the surface markers HLA-A,B,C, CD340, CD49f, and CD151 were expressed on DCX^- cells while CD200 differentially stained DCX^+ cells from other cells in culture. FACS of $CD200^{++}$/HLA-A,B,C^- cells from neural induction cultures, containing 15% neurons resulted in 90% pure population of neurons. Magnetically depleting the neuron induction cultures with antibodies against HLA-A,B,C, CD340 and CD49f enriched these cultures four to five fold for neurons. These methods enable enrichment of neuronal cells from heterogeneous cultures for subsequent downstream analysis.

Poster Board Number: F-1035

ROLE OF WNT AND SONIC HEDGEHOG SIGNALLING DURING TELENCEPHALIC PATTERNING OF HUMAN PLURIPOTENT STEM CELLS DERIVATIVES

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Human pluripotent stem cells can be directed to form neural plate cells (Rosette-Neural Stem Cells: R-NSC) equivalent in adherent culture condition through the synergistic inhibition of both the activin/nodal (SMAD 2/3) pathway and the BMP (SMAD 1/5/8) pathway. During mammals development, Wnt signaling and Hedgehog signaling are known to mediate rostro/caudal of and dorso/ventral patterning of neural plate cells (Backman et al., 2005; Hebert and Fishell, 2008). These typical patterning signals, when applied during R-NSC formation via dual SMAD inhibition, are effective in committing those neural cells to a ventral mesencephalic fate. The progenitor cells generated this way are capable of terminal differentiation into midbrain dopaminergic neurons both in vitro and *in vivo* (Kriks et al., 2011). Here, we explored the same patterning signals in the

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context of telencephalic induction and telencephalic dorso-ventral patterning of hESCs-derived R-NSCs. To investigate the impact of canonical Wnt-signaling on rostro/caudal patterning of hESC-neural derivatives we applied Wnt agonist (WNT3a) or antagonist (DKK-1) early during neural induction of hESCs. Expression of neural, telencephalic and non-telencephalic markers were monitored by QRT-PCR during the first 10 days of differentiation. RNA level of telencephalic markers such SIX3, FOXG1 or OTX2 are significantly higher in R-NSC generated in medium supplemented with DKK-1 while neural crest markers such as SOX10 and PAX3 are reduced indicating that inhibition of WNT signaling promotes telencephalic commitment. Likewise, to explore the impact of SHH signaling on telencephalic dorso/ventral patterning of hESC-neural derivatives in vitro we applied cyclopamine (SHH antagonist) or SHH at increasing concentrations between day 4 and day 28 or between day 10 and day 28 to our human cultures. Supplementation with SHH activates the SHH-pathways as shown by the increased GLI1 and PTCH1 RNA expression levels. SHH treatment induced enhanced ventral telencephalic differentiation as shown by increases in mRNA level and cells number positive for ventral telencephalic markers such as GSX2 and NKX2.1. Parallel reduction in the expression of EMX1, a dorsal marker was observed by QRT-PCR. Interestingly, yield of MGE (NKX2.1+) cells, was maximal either with highest SHH concentration started at day 10 or with intermediate concentration applied earlier starting at day 4. Our results show that maximal enrichment in GSX2+ LGE progenitors in hESC-derived culture is achieved using both early inhibition of Wnt-signaling and moderate activation of SHH signaling. Medium Spiny Neurons (MSN) are the most abundant population of neurons in the striatum and the most vulnerable in Huntington disease (HD). MSN being derived from GSX2+ progenitors in the germinal zone of the lateral ganglionic eminence (LGE), optimal Wnt and SHH modulation is therefore critical for the production of therapeutically relevant graft for HD cell therapy.

Poster Board Number: F-1036

CELL AUTONOMOUS VERSUS NON-AUTONOMOUS EFFECTS OF HUMAN ALPHA-SYNUCLEIN ON NEURITE DEVELOPMENT OF NEW NEURONS IN THE ADULT DG

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α-Synuclein plays diverse roles in the brain: it is physiologically involved in modulating brain plasticity and is a key protein in neurodegenerative diseases, including Lewy body dementia (LBD). We investigated how α-synuclein levels modulate adult neurogenesis and the development and/or maintenance of dendritic arborization and spines in the dentate gyrus (DG), where new neurons are constantly added. α-Synuclein was endogenously present and co-expressed with neural progenitor markers in the human hippocampus, and its levels were increased in LBD, where the numbers of SOX2-positive cells are decreased. We asked if newly generated neurons were also modulated by endogenous α-synuclein and found increased adult neurogenesis in knockout of α/β-synuclein mice. Overexpression of human wild-type α-synuclein (WTS) had a cell-autonomous impact on the survival and dendritic development of newborn neurons. Endogenous α-synuclein expression levels increased the negative impact of WTS on dendrite development. cAMP response element-binding protein (CREB) activation led to a partial rescue of neurite development.

Poster Board Number: F-1037

DISRUPTION AND THERAPEUTIC RESCUE OF AUTOPHAGY IN A HUMAN NEURONAL MODEL OF NIEMANN PICK TYPE C1

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Successful development of effective therapeutic interventions for neurodegenerative diseases will require a deeper understanding of mechanisms of disease initiation and progression. In this respect, an unresolved issue about many neurodegenerative diseases is why neurons are particularly sensitive to defects in ubiquitous cellular processes. One example is Niemann Pick type C1 (NPC1), caused by defects in cholesterol trafficking in all cells, but where neurons are preferentially damaged. Understanding this selective failure is limited by the difficulty of obtaining live human neurons from affected patients. To solve this problem we generated neurons with decreased function of NPC1 from human embryonic stem cells (hESC) and used them to test the hypothesis that defective cholesterol handling leads to enhanced pathological phenotypes in neurons. We found that human NPC1 neurons have strong spontaneous activation of autophagy, but downstream processing of autophagic intermediates is impaired, which induces accumulation of mitochondrial fragments. Mitochondrial fragmentation is an exceptionally severe phenotype in NPC1 neurons compared to fibroblasts, causing abnormal accumulation of mitochondrial proteins. We were able to rescue these abnormal phenotypes in NPC1 neurons by inhibiting autophagy, and by mobilization of cholesterol from the lysosomal compartment. We are currently testing the related hypothesis that imbalanced autophagy in NPC1 induces deficits of mitochondrial function, leading to synaptic pathology and neuronal failure. We also propose that drugs that specifically modulate mitochondrial targeting to the autophagic pathway may rescue NPC1 neurons from the effects of imbalanced mitophagy without affecting turnover of other autophagic cargoes. Our data highlight the central role that autophagy failure caused by lysosomal cholesterol accumulation plays in the selective neuronal failure observed in NPC1, and provides the first example of a process that causes preferential neuronal defects in this devastating childhood neurodegenerative disease. Additionally, our approach establishes a cell-based platform for the high-throughput screening of potential therapeutic compounds that can revert accumulation of cholesterol, lysosomes, mitochondrial fragments, and autophagic intermediates in NPC1 and related neurodegenerative diseases.

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Poster Board Number: F-1038

ENHANCED NEURONAL DIFFERENTIATION OF HUMAN NEURAL STEM CELLS ON TITANIUM-GRAFTED NANOSCALE PATTERN SURFACES

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Human neural stem cells (hNSCs) with the capacity of differentiating into mature neurons can provide a novel therapeutics for the treatment of neurodegenerative disease and neuronal injury. Biomaterial substrates and scaffolds for hNSC culture and transplantation have been reported to be able to modulate differentiation of hNSCs by providing cell-matrix interaction. Particularly, the surface topography at nanoscale level may significantly change differentiation of hNSCs. Here, we report the nanotopographical effects of polymer substrates on neuronal differentiation of hNSCs. The polyurethane acrylate (PUA) nanopatterned surface was grafted with titanium (Ti) for potential electrical stimulation by initiated chemical vapor deposition technique. The culture of hNSCs on Ti-coated PUA groove nanopatterned surfaces promoted elongation and alignment of hNSCs along with the axis of patterned surfaces, as indicated by F-actin cytoskeleton (phalloidin) staining. The hNSCs on Ti-coated PUA flat surfaces without groove nanopatterns did not exhibit such aligned and elongated cell morphology. Immunocytochemical staining for neuronal marker (neuronal Class III β-Tubulin: Tuj1) revealed that cell bodies aligned along the groove patterns were stainedpositively against Tuj1, indicating the enhanced neurite extension from hNSCs on the groove patterns. In summary, we found that differentiation of hNSCs, especially, into neuronal lineage cells was enhanced by nanoscale surface topography. In the future, we will consider electrical stimulation of Ti-grafted nanopatterned surface because electrical signals may further promote neuronal differentiation of stem cells. Acknowledgement: This study was supported by grants (2010-0020409 and 2011-0027538) funded by the National Research Foundation of Korea.

Poster Board Number: F-1039

A HUMAN IPSC MODEL OF AUTISM SPECTRUM DISORDERS DUE TO HAPLOINSUFFICIENCY OF MEF2C

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Autism Spectrum Disorders (ASD) are a complex and poorly understood group of developmental disorders associated with abnormalities in neural development. Although our understanding of ASD is still very limited, several genetic and environmental factors have been causally linked to their etiologies. Recently, dominant mutations localized exclusively to myocyte enhancer-binding factor 2C (MEF2C) have been identified in a number of ASD patients carrying 5q14 microdeletions. These studies also indicate that MEF2C deletions are relatively frequent, occurring in as many as 1.1% of patients with autosomal dominant ASD and related neurological disorders. Previously, our group had discovered Mef2c and reported that mice null for this transcription factor at the neural progenitor/stem cell (NPC) stage in the CNS manifest impaired behaviors reminiscent of ASD in conjunction with reduced synaptic number and function. Consistent with these results, we recently observed that Mef2c-heterozygous (het) mice also show behavioral and electrophysiological phenotypes resembling ASD, resembling human patients haploinsufficient for Mef2c. In the current study, using patient-specific iPSCs, we aim to understand the neurodevelopmental deficits associated with Mef2c haploinsufficiency and its relationship to ASD etiology. Towards this end we have generated iPSC lines from patients carrying deletions in Mef2c. We have directed the differentiation of these iPSCs to neural progenitors and subsequently to mature neurons. We are analyzing the survival, self-renewal and differentiation capabilities of the Mef2c-haploinsufficient iPSCs as well as the electrophysiological phenotype of the iPSC-derived neurons compared to wild type controls.

Poster Board Number: F-1040

DIRECT LMX1A PROTEIN TRANSDUCTION; AN EFFICIENT APPROACH TO PRODUCE HIGH PURE AND SAFE DOPAMINERGIC NEURONS FROM HUMAN EMBRYONIC STEM CELLS

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Human embryonic stem cells (hESCs) are pluripotent cell lines with the potential to form any human cell type and can be propagated *in vitro* in an undifferentiated stage. Because of their exceptional properties, hESCs have tremendous potential to be used for developmental biology study, drug screening, functional genomics applications, and regenerative medicine. For all applications of hESCs, pure populations of selected cell types will be a likely prerequisite. Here, we present a protein transduction based method as a safe approach to increase the efficiency of hESCs differentiation into Dopaminergic (DA) neurons using direct LMX1A protein transduction. Royan H6 cell line of hESC was induced to neural ectoderm using Noggin and RA treatment for six days. Then neural progenitors treated with FGF8 and SHH for differentiation toward DA progenitors in the presence of Recombinant LMX1A protein fused to TAT and without LMX1A for additional 10 days. LMX1A protein was successfully translocated in the nucleus in neural progenitors. Quantitative RT-PCR and immunofluorescence analyses showed that LMX1A protein transduction could enhance DA neurogenesis more than two folds compared to the control cells. Flow cytometry analysis significantly revealed more expression of PITX3 and GRIK2 in the test group cells population compared to the control group. These progenitors could differentiate to mature DA neurons, which express TH and Nurr1. The analysis of cell membrane channels specifically sodium channels, and transplantation into the animal models of Parkinson's disease are in progress.

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Poster Board Number: F-1041

HUMAN IPS CELL-DERIVED NEURAL PROGENITORS ALLEVIATE STROKE-INDUCED FUNCTIONAL IMPAIRMENTS.

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Induced pluripotent stem cells (iPSC) are an appealing source for cell replacement therapy in acute brain lesions. However, iPSC associate embryonic stem cell (ESC)-like properties, selfrenewal and pluripotency, with genetic and epigenetic modifications, which, altogether, introduce significant challenges for clinical application. Here, we used a combination of functional, imaging, and histological techniques to evaluate the potential of hiPSC for regenerative therapy in stroke. The fate and functional effects of neural precursor cells (NPC) derived from hiPSC were compared to hESC-NPC in a rat model of focal stroke. Significant recovery of impaired functions was observed from one month post-transplantation. However, functional recovery was temporally dissociated from the development of neurons with adequate region-specific phenotypes, i.e. GABAergic striatal neurons, in the graft, suggesting the existence of non cell-autonomous mechanisms in the first weeks following transplantation. With time, grafted neurons developed axons that projected into the host substantia nigra, in correlation with significant reduction of ischemia-induced atrophy of this structure. These results show that hiPSC-NPC bear similar potential for regenerative medicine in stroke than hESC. They also show that the mechanisms of action of hiPSC-NPC include rapid, likely paracrine, effects, and delayed effects linked to the maturation and integration of grafted cells.

Poster Board Number: F-1042

DYNAMICS OF OLIGODENDROCYTE TURNOVER IN HUMANS

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Oligodendrocytes are the myelinating cells of the CNS and are essential for proper brain function as they produce the myelin sheath, which is critical for fast axonal conduction and survival of the axons. Recently, studies in experimental animals have shown that there is a continuous generation of oligodendrocytes in the adult brain. However, to what extent this event occurs in the adult human brain remains elusive as methods employed in experimental animals, such as paradigms with labeled nucleotide analogs are not possible to apply in humans. In order to study oligodendrocyte turnover we are using a novel strategy that enables retrospective birth dating of cells in humans. This strategy takes the advantage of the integration of 14C, derived from nuclear bomb testing during the Cold War, into genomic DNA. The level of 14C in the genomic DNA will reflect the atmospheric level at the time of cell birth, thus making it possible to establish the age of human cells and thereby directly estimate turnover in the adult human brain. We find evidence for generation of oligodendrocytes throughout adulthood. We find that oligodendrocytes are in average 3.37±2.5 years younger than the individual. The prospect of ongoing oligodendrogenesis is of particular interest as this is the main cell population affected in multiple sclerosis. Our finding that oligodendrocytes are generated in the adult brain point to a potential target for new therapeutic interventions aiming at affecting this process.

Poster Board Number: F-1043

SYSTEM X_C^- MEDIATES MIGRATION AND VIABILITY OF HUMAN NEURAL STEM CELLS AND EXHIBITS ANTI-APOPTOTIC EFFECTS AGAINST ROS-INDUCED SIGNALING FACTORS

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Recent progress in stem cell biology includes therapeutic regenerative strategies to replace lost neural cells in damaged CNS and promote host tissue regeneration via stem cell transplantation. Research also supports harnessing the inherent tropism of neural or mesenchymal stem cells to deliver therapeutic agents directly to invasive brain tumor cells, resulting in tumor-localized chemotherapy. We have previously shown that a stable, immortalized human neural stem cell (NSC) line, HB1.F3, migrates to glioma conditioned media *in vitro* and selectively migrates to orthotopic human glioma in immunodeficient mouse models. Here we show that the HB1.F3 NSCs express high levels of the heterodimeric cystine/glutamate antiporter known as system XC- which functions as a sodium-independent electroneutral transporter for cystine influx coupled to glutamate efflux. System XC- is found predominantly in the brain with distribution mainly in neurons and glial cells and in areas that mediate transport of nutrients between the brain parenchyma and surrounding tissue. The transporter is responsible for maintaining redox homeostasis by importing cystine into cells, where it is reduced to cysteine and used for the synthesis of the antioxidant glutathione and for protein synthesis. We demonstrate that inhibition of system XC- accomplished pharmacologically using sulfasalazine increased intracellular reactive oxygen species (ROS) and early apoptosis of the NSCs in a dose dependent manner. System XC- has been shown to be neuroprotective when over-expressed in non-neuronal cells and when inhibited causes apoptosis in brain tumor cells. NSCs are vulnerable to oxidative injury due to the hypoxic environment in the brain, and maintenance of glutathione levels may be critical for cell survival and proliferation. Inhibition of system XC- also attenuated the migration of the HB1.F3 cells to glioma conditioned media in a dose dependent manner. An excess of extracellular glutamate which also blocks cystine uptake via system XC- showed similar effects. We show for the first time that system XC- plays a significant role in human NSC migration and survival.

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Poster Board Number: F-1044

MODELING LISSENCEPHALY WITH PATIENT-DERIVED IPSCS

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One of the most severe neurological disorders, Classical Lissencephaly (LIS), arises when developing neural progenitor cells fail to migrate to stratify the cerebral cortex lamina properly. The process of neural migration in humans begins early, at gestational age 7 weeks. Hence, patients with LIS are not typically diagnosed with the disorder until after the cortex has incorrectly developed, severely limiting treatment. Potential future therapies for LIS, such as genetic prenatal diagnosis or manipulation of defective gene regulatory networks during development, require extensive understanding of the genetic, cellular, and molecular mechanisms controlling neural migration. Traditionally, this knowledge was obtained by analyzing gene-deficient mice; however, phenotype comparisons between *LIS1*-deficient LIS patients and *Lis1* mutant mice have shown that the genetic pathways underlying neural migration have redundancy in mice compared to humans. In addition, defects in neural migration may be more readily detectable in humans versus mice due to the extended distance neurons must travel in a human cortex to reach their target locations. Therefore, in order to understand the underlying cause of LIS and improve patient therapies, I have generated a patient-specific, induced pluripotent stem cell derived, model for this disorder. This new model is now being utilized to investigate the aberrant mechanisms underlying LIS in five genetically distinct multiplex families with novel causative gene mutations.

Poster Board Number: F-1045

HUMAN EMBRYONIC AND FETAL NEURAL STEM CELLS COMPARED IN A PRIMATE MODEL OF PARKINSON'S DISEASE

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Parkinson's disease (PD) was one of the first neurological diseases to be studied for potential replacement of diseased neurons, beginning with fetal substantia nigra dopamine (DA) precursor cells in the late 1970's. Since then, much work has been performed utilizing fetal precursor cells, including human clinical trials which were met with limited success and identified several problems. The discovery of neural stem cells (NSCs), embryonic and induced sources of neural cells have provided new avenues for creating replacement cells to restore function in PD. There has also been great progress with learning the required gene expression, growth factors, and culture conditions for differentiating cells into apparent DA neurons. However, with the exception of a recent report [Kriks et al. Nature 480:547-51, 2011], DA neurons differentiated and characterized in culture have not produced long-lasting midbrain specific DA neurons *in vivo* in rodents or monkeys, and there have been only brief pilot reports of convincing functional improvement. The objective of our studies has been to test a number of cell types, which have shown promise for PD therapy. The strongest candidates were then investigated for cell survival and dopamine neuronal phenotype after stereotaxic placement into the nigrostriatal system in the best available model of human PD: MPTP (methyl-4-phenyl-1,2,3,6-tetrahydropyridine)-exposed monkeys. We derived several stem cell candidates (listed below), using published methods, and then characterized these cells in vitro before implanting them into immunosuppressed MPTP monkeys. The methods for monkey studies were described in a prior study of human fetal NSCs [Redmond et al., PNAS 104: 12175-80, 2007]. Here we derived neural cells from human embryonic stem cells (hESCs) (Lines H1 & H9) and compared them with human NSCs derived directly from the fetal CNS with or without augmentation by AAV5-transduced glial-derived neurotrophic factor (GDNF) injections. The results demonstrated that cells from these sources had properties associated with DA neurons in culture, and all survived in substantial numbers *in vivo*. Concordant with our previous findings, undifferentiated multipotent fetal NSCs, with or without AAV-GDNF neurotrophic injections, showed small numbers of tyrosine hydroxylase positive cells after up to 1-year *in vivo*. The H1 hESC-derived cells showed excellent survival, apparent integration into the host, a predominantly neuronal phenotype, but were not TH+. The H9 hESC-derived cells and NSCs, which had been differentiated in a glial conditioned medium with fibroblast growth factor-2 (FGF-2) and leukemia inhibitory factor (LIF), displayed a larger percentage of TH+ cells after 2 months *in vivo*. None of the cell types produced overgrowth or tumors. Our conclusion is that neural cells characterized as dopaminergic neurons in culture may not in fact be identical to midbrain fetal DA precursor cells, which survive long-term *in vivo* and maintain sustained midbrain dopaminergic function. Actual survival, DA phenotype, integration, dopamine release, and long-term functional improvement in a primate PD model is necessary to propel any stem cell based therapy to a clinical trial. Supported by California Institute of Regenerative Medicine, M. J. Fox Foundation, NIH, Axion Research and St. Kitts Biomedical Research Foundations. Also by the Connecticut Stem Cell Research Grants Program. *Co-corresponding authors.

Poster Board Number: F-1047

DERIVATION OF MULTIPOTENT HUMAN NEURAL PLATE BORDER STEM CELLS UNDER CHEMICALLY DEFINED CONDITIONS

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The neural tube and neural crest lineages are specified during gastrulation from the neural plate. Sonic hedgehog induces ventral neural tube character, whereas WNT signaling specifies formation of neural crest cells. Here we report that the combination of both signals enables the derivation and propagation of human neural epithelial cells that most closely resemble neural plate border stem cells (NPBSCs). These NPBSCs can be clonally directed to differentiate into neural tube and neural crest lineages, including both peripheral neurons and mesenchymal cells, which is a property so far only matched by pluripotent stem cells. NPBSCs are responsive to developmental morphogens and form lineages such as motor neurons with about 50% efficiency, higher than pluripotent stem cells treated with exogenous growth factors. Therefore, NPBSCs are a unique and powerful biological system and tool that will facilitate

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large-scale disease modeling and high-throughput phenotypic screening as well as studies of early human development not otherwise possible.

Poster Board Number: F-1048

MENINGEAL CELLS INDUCE ASTROCYTE DIFFERENTIATION OF NEURAL STEM CELLS

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The major cell types in the central nervous system, neurons and glial cells (astrocytes and oligodendrocytes), are generated from common neural precursor cells (NPCs). NPCs differentiate only into neurons at midgestation and gradually begin to differentiate into glial cells during late gestation. The mechanisms driving this stepwise process are now becoming apparent. We have previously shown that Notch ligands are expressed on committed neuronal progenitors and immature neurons in mid-gestational telencephalon, and that these ligands activate Notch signaling in adjacent NPCs, leading to demethylation of astrocytic genes. This demethylation enables NPCs to acquire the potential to become astrocytes in response to astrocyte-inducing cytokines, such as IL-6 family cytokines that activate transcription factor STAT3. However, the cells which secret IL-6 family cytokines remain to be identified. In this study, we focus on the meningeal basement membrane (BM) that covers cortex as a candidate for the source of IL-6 family cytokines, since NPCs extend radial fibers that are anchored at the meningeal BM. We found that NPCs differentiated predominantly into astrocytes when co-cultured with meningeal cells. Furthermore, conditioned medium (CM) collected from the culture of meningeal cells also induced astrocyte differentiation, suggesting that astrocyte-inducing factor(s) secreted from these cells. Luciferase assay using the typical astrocytic marker *gfap* gene promoter revealed that CM alone could activate the promoter. However, expression of the dominant negative form of STAT3 and treatment with RX435, which is an inhibitory antibody specific for critical IL-6 family cytokine's receptor component gp130 suppressed the *gfap* promoter activation induced by the CM. Taken together, it is conceivable that meningeal cells induce astrocyte differentiation of NPCs by secreting IL-6 family cytokines during brain development.

Poster Board Number: F-1049

CATALOGUING THE FUNCTION OF LONG NONCODING RNAS EXPRESSED IN MOUSE NEURAL STEM CELLS IN THE NEUROGENIC NICHES OF THE ADULT BRAIN

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Recently, long non-coding RNAs (lncRNAs) have been shown to integrate into the molecular machinery controlling pluripotency and lineage specification of embryonic stem cells, but comparatively little work has been done to address lncRNA function in an adult stem cell system. The adult mouse brain contains two well-characterized neurogenic niches: the subventricular zone (SVZ) and the subgranular zone of the hippocampus (SGZ). These niches generate terminally differentiated neurons and glia of the olfactory bulb (OB) and hippocampal dentate gyrus. To gain greater insight into the functions of lncRNAs in these adult stem cell systems, we performed high throughput cDNA sequencing and ab initio reconstruction of all poly-adenylated transcripts expressed in these neurogenic brain regions. Using a non-coding RNA annotation pipeline, we identified a total of 2109 unique lincRNA transcripts expressed from 1338 loci. Our dataset, combined with previously published lincRNA annotations, provides a comprehensive list of ~6000 lncRNA transcripts. These transcripts can be alternatively spliced and show heterogeneous and region-specific expression across several regions of the adult brain, including cortex, striatum, and hypothalamus. A subset of ~2000 lncRNAs are located within 5kb of a protein-coding gene promoter. This set of protein-coding genes is specifically enriched for key homeobox and forkhead transcription factors, as well as signaling pathways essential for brain development and patterning. We identified a subset of lncRNAs that are highly expressed in neurogenic brain regions, and we demonstrate that their expression levels can be modulated by neurogenic cues in a cell-culture system that recapitulates SVZ neurogenesis. By comparing genome-wide chromatin-state maps of embryonic stem cells and SVZ-derived neurospheres, we define sets of protein-coding genes and lncRNAs that correlate with adult neurogenesis. Through gene coexpression analysis, we infer lncRNA function by placing them within known gene-expression modules. This work provides a comprehensive look at lncRNA expression throughout an adult tissue, including two stem cell niches. It lays the foundation for ongoing studies that infer function through analysis of gene neighbors, chromatin-state maps, and co-expression module analysis. Other ongoing work includes a global look at lncRNA expression changes throughout neuronal differentiation using an SVZ cell culture system with custom-designed microarrays, and validation of lncRNA function using shRNA constructs directed against rationally chosen lncRNA candidates.

Poster Board Number: F-1050

NEURAL CELLS DIFFERENTIATION *IN VITRO* FROM MOUSE AMNIOTIC EPITHELIAL STEM CELLS

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OBJECTIVE: The goal of this study is investigation of the inductive effect of brain extract on amniotic epithelial stem cells (AESCs) and the ability of AESCs to differentiate into neural cells *in vitro*. METHODS: AESCs were isolated from mouse NMRI amnion on E14-16 and cultured in Dulbecco's Modified Eagle's Medium (DMEM) which supplemented with 10% fetal bovine serum (FBS). Moreover the viability of AESCs was determined by exclusion of trypan blue dye. AESCs achieved >90% confluency in 4 days and the cells were cultured in neural differentiation media (DMEM, 1% FBS, 50µM brain extract) and then let them to differentiate to neural cells for 21 days. For induced cells the Flowcytometry and immunocytochemistry analysis have been used for assessing specific neural cells markers Nestin, microtubule-associated protein (MAP-2). RESULTS: Flowcytometery analysis showed that the percentage of neuronal marker Nestin expressing on the seventh day after induced by brain extract, was 88.53%. Also the results of immunocytochemical staining have showed the expression of MAP-2. CONCLUSION:The present study demonstrated that brain extract due to having some specific growth factors plays a crucial role in the differentiative behaviour of AESCs. Transdifferentiation capacity of AESCs into neurons, clues the usage of AESCs in stem cell therapy for neurodegenerative diseases. Keywords: Amniotic epithelial stem cell, brain extract, differentiation, neural cells, neural markers,

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Poster Board Number: F-1051

COMPARATIVE STUDY OF SUB-ACUTE AND CHRONIC NEURAL STEM CELL TRANSPLANTATION FOR SPINAL CORD INJURY IN ADULT MICE

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Transplantation of neural stem cells (NSCs) at the sub-acute phase of spinal cord injury (SCI) has been shown to promote functional recovery, whereas NSCs transplantation at the chronic phase of SCI did not promote functional recovery. However, the differences in the survival, the differentiation phenotype and the distribution of the grafted cells between the sub-acute and chronic NSCs transplantation still remain unclear. In this study, to determine these issues we performed sub-acute and chronic NSCs transplantation for SCI in adult mice. E14.5 fetal brains-derived NSCs were prepared from novel transgenic mice expressing both luminescent and fluorescent proteins as neurospheres. Contusive SCI was induced by the IH impactor at Th10 level in adult female C57/BL6J mice. Sub-acute and chronic NSCs transplantation into the lesion epicenter were performed at 9 days and 6 weeks after SCI, respectively. In the vehicle control, only PBS was injected instead of NSCs at each time-point. The survival rate of grafted cells was evaluated by *in vivo* bioluminescent imaging. Motor function of hindlimbs was assessed weekly by Basso Mouse Scale (BMS). Rota-rod testing, treadmill gait device (DigiGait system) and then electrophysiological analysis were performed at 6 weeks after each transplantation, followed by histological examinations. Quantitative analysis using bioluminescent imaging revealed no significant difference in the survival rate of grafted cells between the sub-acute and chronic groups at all the examined time-points. Immunohistochemical analysis showed that the grafted cells differentiated into three neural lineages in the both groups. There were no significant differences in the differentiation patterns of grafted NSCs between the two groups. Interestingly, the grafted cells were distributed at the epicenter as well as the rostral and caudal sites in the sub-acute group, whereas the grafted cells existed just around the lesion epicenter in the chronic group. While the sub-acute group exhibited significantly better functional recovery than the vehicle control group, the chronic group did not show any functional recovery after the chronic transplantation. Furthermore, electrophysiological analysis revealed that motor evoked potential (MEP) waves with higher amplitude were observed in the sub-acute group than the chronic group, and no waves were detected in the sub-acute and chronic vehicle control groups. Taken together, these findings suggested that the chronic NSCs transplantation did not promote functional recovery despite the similar survival and differentiation phenotype of the grafted cells as the sub-acute NSCs transplantation. Since the robust glial scar existed at the lesion site at the chronic phase of SCI, the grafted cells were localized just around the lesion epicenter in the chronic group. Combined therapy of suppression of axonal growth inhibitor and/or rehabilitation with chronic NSCs transplantation could be critical to promote functional recovery after chronic SCI.

Poster Board Number: F-1052

DIFFERENTIAL EXPRESSION OF FABP7 AND FABP5 IN MOUSE OLIGODENDROCYTE LINEAGE CELLS

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Polyunsaturated fatty acids (PUFAs) have important metabolic, structural and signal transducing roles. PUFAs are essential for normal development of the brain and their preventive and therapeutic roles have been shown in neuropsychiatric and neurodegenerative diseases such as schizophrenia and multiple sclerosis. Fatty acid binding proteins (FABPs) are key intracellular molecules in the uptake, transportation and storage of PUFAs and in mediating their signal transduction and gene regulation activities. FABP7 and FABP5 are abundantly expressed in the developing brain, suggesting their role in proliferation /differentiation of neural stem cells by regulating fatty acid homeostasis. However, little is known about their expression pattern in the oligodendrocyte lineage cells, which are important targets of cell therapy and regenerative medicine. In this study, we evaluated the expression of FABP7 and FABP5 in the oligodendrocyte lineage cells in the cortex and corpus callosum of the adult mice, and in the primary culture of mouse oligodendrocyte progenitor cells (OPCs) by immunofluorescent staining with olig2 (common oligodendrocyte lineage marker), NG2 (OPC marker), and CC1 (marker for mature oligodendrocytes). In the cortical gray matter, majority of olig2+ cells were FABP7+ but a few of them were FABP5+. Conversely, in subcortical white matter and corpus callosum a minor subpopulation of olig2+ cells were FABP7+ and majority of them were FABP5+. NG2+ OPCs in the cortex and corpus callosum expressed FABP7 but did not express FABP5. In contrast, CC1+ oligodendrocytes in the cortex and its underlying white matter expressed FABP5 but did not express FABP7. In the mouse primary OPC culture, the majority of olig2+ cells or NG2+ OPCs were FABP7+ FABP5-, which is in accordance with our *in vivo* results. In addition, FABP5 expression was detected in CC1+ mature oligodendrocytes where no FABP7 expression could be detected. Expression of FABP3, another known brain- expressed FABP, was not detected in the oligodendrocyte lineage cells. By showing a reciprocal expression of FABP7 and FABP5 between OPCs and mature oligodendrocytes, our present data introduce novel markers for oligodendrocyte lineage, and suggest the involvement of FABPs in the regulation of their proliferation/differentiation. These findings provide new insights into lipid homeostasis in the process of differentiation of oligodendrocyte lineage cells which may support the preventive and therapeutic roles of PUFAs in neuropsychiatric and neurodegenerative diseases. We are currently studying the role of FABP7 and FABP5 on proliferation and differentiation of OPCs in the normal and pathological conditions by using FABP7-KO and FABP5-KO mice.

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Poster Board Number: F-1053

IDENTIFICATION OF MACROPHAGE MIGRATION INHIBITORY FACTOR (MIF): A NOVEL FUNCTIONAL MOLECULAE PROMOTING CELL SURVIVAL AND PROLIFERATION OF MOUSE NEURAL STEM/ PROGENITOR CELLS

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Identification of molecules that promote cell survival and/or proliferation of neural stem/ progenitor cells (NSPCs) may be utilized in future therapeutic applications. In a previous study, we showed that murine dendritic cells (DCs) increase the number of NSPCs in vitro and *in vivo*. In the present study, we identified macrophage migration inhibitory factor (MIF) as a novel factor that can support proliferation and/or survival of NSPCs in vitro. MIF is secreted by DCs and NSPCs; however, MIF function in the normal brain has previously remained largely unknown. It was previously shown that in macrophages, MIF binds to a CD74-CD44 complex. Here, we observed the expression of MIF receptors in mouse ganglionic eminence (GE)-derived neurospheres using flow cytometry in vitro. We also found CD74 expression in the GE of E14 mouse brains, suggesting that MIF plays a physiological role *in vivo*. MIF increased the number of primary neurospheres, and secondary neurospheres supporting self-renewal ability. In contrast, retrovirally-expressed MIF shRNA and MIF inhibitor (ISO-1) suppressed primary and secondary neurosphere formation, as well as cell proliferation. MIF knock-down by shRNA increased caspase 3/7 activity. MIF increased phosphorylation of Akt, Erk, AMPK, and Stat3 (Ser727), neurosphere cell surface expression of Glut1, and gene expression of Hes3 and Egfr which are thought to support cell survival, proliferation and/ or maintenance of NSPCs. Furthermore, MIF up-regulated gene expression of Sox6 in these neurospheres, which can support the self-renewal of GE-derived neurospheres. MIF is also revealed as a chemo-attractant for NSPCs in the in vitro chemochamber migration assay and brain slice culture system. Taken together, MIF can induce NSPC proliferation and maintenance by using multiple-signaling pathways synergistically, and it may be a potential therapeutic factor capable of NSPC activation for the treatment of degenerative brain disorders.

Poster Board Number: F-1054

GENERATION OF DOPAMINERGIC NEURONS DIRECTLY FROM MOUSE FIBROBLASTS AND FIBROBLAST-DERIVED NEURAL PROGENITORS

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Loss of midbrain dopaminergic (DA) neurons is a pathological feature of Parkinson's disease (PD). Recent advances in the reprogramming research field indicate that it may be possible to convert somatic cells into mature dopaminergic neurons relevant in disease modeling and/or replacement therapies. In this study, we have successfully converted mouse fibroblasts into DA neurons by two separate routes. One is to introduce into fibroblasts DA lineage specific factors combined with Ascl1 and Brn2, the two induced neuron (iN) factors that can convert fibroblasts to mature neurons; and the other is to first reprogram mouse fibroblasts into neural progenitors that possess the potential of DA neuron specification. Two different groups of DA lineage specific factors (Foxa2 and Lmx1a; or Nurr1, Lmx1b and Otx2), combined with Ascl1 and Brn2, can directly induce mouse embryonic fibroblasts (MEFs) into DA neurons. We have previously shown that sertoli cell, a mesoderm-derived terminally differentiated cell, can be reprogrammed directly to a neural stem cell-like cell by defined factors. Here, by introducing the same gene cocktail, tail tip fibroblasts (TTFs) can also be induced to a multipotent state resembling neural progenitor cells. The induced neural progenitor cells (iNPCs) are capable of self-renewal and differentiation into neurons and glia in vitro, as well as surviving transplantation and giving rise to mature neurons *in vivo*. Furthermore, iNPCs can commit to DA neuronal lineage after treatment with the patterning factors, sonic hedgehog (Shh) and fibroblast growth factor-8 (FGF-8). The DA neurons obtained from either direct conversion or fibroblast-derived progenitors show functional membrane properties and express En1 and Pitx3, indicating a midbrain DA neuronal identity. The findings of this study may provide a tool for disease modeling and a new therapeutic strategy for treating PD.

Poster Board Number: F-1055

CHARACTERIZATION OF ADULT MOUSE TISSUE SPECIFIC STEM/PROGENITOR CELLS IN INFERIOR COLLICULLUS

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To investigate the keys of treatments for the deaf patients that have auditory damages, we tried to isolate stem/progenitor cells from inferior colliculus in the auditory pathway and analyzed the cell characterization and gene expressions. To identify the slow cycling cells suggesting the possibility of stem cell, BrdU was injected to hypodermic 2 times/day for 3 days continuously and sacrificed after 8 weeks. The results of immunohistochemistry showed that a few cells were identified as BrdU+ and ABCG2+ double positive cells in the section of inferior colliculus. Spheroid formation was detected when the inferior colliculus cells were grown in serum-free, non-adherent culture. Furthermore, we assumed that it was possible to purify stem cells as side population (SP) cells, inferior colliculus cells were isolated from 3 weeks age of mice and stained with Hoechst 33342. After staining, SP cells were sorted as a negative fraction. The population of SP cells was about 1-2 % of total inferior colliculus cells. Additionally, in order to characterize the cell characterization of SP cells, we used microarray techniques by Mouse oligo Microarray (Agilent technologies). The results showed that the up-regulated genes in SP cells were included some specific markers of stem cells, ABCG2 etc. We analyzed the gene expression of SP cells by RT-PCR. The results showed that some specific markers of stem/progenitor cells, such as Oct-3/4, Sox2, and ABCG2, were over-expressed in SP cells compared with main population (MP) cells. Currently we are going to study whether these cells have the multipotential ability as stem/progenitor cells.

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Poster Board Number: F-1056

EPIGENETIC REGULATION OF MOUSE NEURAL STEM CELL DIFFERENTIATION AND FUNCTIONAL BRAIN DEVELOPMENT

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Precise regulation of neural stem cells (NSCs) during development is important for brain architecture and function. Much attention is recently being paid to epigenetic regulation of the NSC-differentiation for understanding brain development and tackling neural diseases, because solely DNA sequence-based approaches may not fully explain normal and disease conditions in the brain. It had been a long standing question how astroglial differentiation is prevented until the late gestational stage even though astrogliogenic cues and their downstream signaling components are present in early and mid-gestational stages. We demonstrated that DNA methylation is a critical determinant in the developmental stage-dependent regulation of astroglial differentiation. We demonstrated that the cytosine residue in the CpG dinucleotide present in the critical DNA element for the binding of a transcription factor STAT3, a downstream target of astroglial differentiation cytokines, in an astroglial gene promoter is highly methylated until the mid-gestational stage and then is demethylated as the brain develops. We recently showed that the expression level of *Tet3*, whose gene product TET3 converts the 5-methylcytosine to 5-hydroxymethylcytosine and then leads to either demethylation of this cytosine and/or hindrance of access of gene-silencing protein(s), increased in mouse brain in accordance with development. Furthermore, forced expression of TET3 in the culture of mid-gestational neural stem cells which do not normally respond to astrogliogenic cytokines rendered these cells responsive to such cell-external cues. Conversely, knock-down of *Tet3* expression inhibited astrocyte differentiation of late-stage neural stem cells. Mouse models established in consideration of epigenetic regulation of the brain can be useful for understanding disease mechanisms and also for therapeutic drug development to tackle neurological disorders. *gasc1* (*gene amplified in squamous cell carcinoma 1*) encodes a histone H3 lysine 9 (H3K9) demethylating enzyme. Alteration of the H3K9 methylation status is known to be related to the control of gene expression. We show that *gasc1* is expressed in post-mitotic neurons in the brain. A mutant mouse strain was developed by a gene-trap insertional mutagenesis in this gene. Homozygous mutant mice exhibited significantly low expression of *gasc1*. Histology of the homozygous mutants showed no obvious defects, by the fall in the expression of *gasc1*, in the brain and other organs during development and up until adulthood. However, in a rotarod test, the impaired acquisition of skilled behavior occurred in the mutant mice. The mutants also displayed hyperactivity and enhanced locomotion in the open field test compared with wild-type control mice. In conclusion, we suggest a novel epigenetic regulation of astrogliogenesis and also propose a new mouse model with an epigenetic modification defect whose phenotype resembles human neurological/psychiatric symptoms.

Poster Board Number: F-1057

NEURAL CREST-DERIVED CELLS PRODUCE MULTIPLE NEURAL CELL TYPES IN THE MOUSE FOREBRAIN

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Neural crest cells originate from the neural folds and migrate into the various embryonic regions where they differentiate into multiple cell types. A population of neural crest-derived cells (NCDCs) invades back into the developing forebrain to differentiate into microvascular pericytes, but less is known regarding the existence of NCDCs in the postnatal brain. Using a transgenic mouse line in which NCDCs are genetically labeled with EGFP, we observed 1) that NCDCs invaded into the telencephalon with endothelial cells at embryonic day (E) 9.5 and 2) that a majority of these NCDCs expressed pericyte markers, i.e., PDGFRβ and NG2, at E11.5. Interestingly, approximately 5% of these NCDCs were NG2-negative and p75-positive; the latter of which is a neural crest cell marker. Proliferation and pericyte differentiation appeared to occur in the specific mesenchymal region where blood vessels and the basement membrane of the telencephalon were in close contact. Postnatally, NCDCs were observed to be scattered within the forebrain as multiple cell types, including not only pericytes but also neurons, astrocytes, oligodendrocytes, and oligodendrocyte precursors. Notably, the NCDCs that expressed neural stem/progenitor cell markers were observed in several areas, including those that are involved in adult neurogenesis, i.e., on the walls of the lateral and third ventricles and the choroid plexus. Taken together, our data suggest that NCDCs invade the developing forebrain, differentiate into various cell types, and may even retain multipotency in postnatal stages.

Poster Board Number: F-1058

COMPARATIVE STUDY OF NEURAL STEM CELL TRANSPLANTATION FOR SPINAL CORD INJURY IN BETWEEN YOUNG AND AGED MICE

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Introduction: To reflect the transition to aging society, it has been pointed out the number of patients with SCI over 60 years old tends to increase. Therefore, establishment of the treatment for elderly patients with SCI is an urgent need. In this study, we compared the therapeutic effects of Neural stem cells (NSCs) transplantation for SCI in young adult (2-month-old) and aged (15-month-old) mice. Methods: SCI was induced by IH impactor (70kDyn) in both young and aged mice, fetal brain derived NSCs of CAG-ffluc-venus Tg mice were transplanted into the lesion epicenter at 9 days after SCI (young- and aged TP groups). In the vehicle control group of both young and aged mice, PBS was injected into the lesion site (young- and aged -control groups). Behavioral analysis was performed using BMS score and the survival of the grafted cells was examined using bioluminescense imaging. For histological analysis, the spinal cords of two groups were resected and analyzed by immunohistochemistry. Results: The BMS score of the aged-control group was significantly lower than that of the young-control group. Interestingly, compared to the aged-control group, significantly better functional recovery was observed in the aged-TP group,

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which was similar to that observed in the young-TP group. The survival rate of grafted NSCs in the aged-TP group was significantly higher than that in the young-TP group. In the aged-TP group, the grafted NSCs predominantly differentiated into neurons and less into astrocytes. Furthermore, RT-RCR analysis of inured spinal cord harvested at 2 weeks after SCI revealed lower gene expression of sema3a, robo3, IL-6 and CTNF, and also higher gene expression of hepatocyte growth factor in the aged-control group compared to the young-control group. Conclusion: Taken together, the difference in the microenvironment between young and aged SCI plays a role in functional recovery after NSCs transplantation. We conclude that advanced age does not prevent a beneficial response to NSCs transplantation following experimental spinal cord injury.

Poster Board Number: F-1059

DEFICIENCY OF DGCR8 GENE, A POTENTIAL GENE FOR 22Q11 DELETION SYNDROME, DECREASES CELL PROLIFERATION AND NEUROGENESIS IN THE ADULT MOUSE DENTATE GYRUS

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MicroRNAs (miRNAs) represent a class of small non-coding RNAs that are key regulators of many cellular events. Although recent studies have suggested that the functional significance of adult neurogenesis in hippocampal-dependent memory, the biological relevance of miRNAs in adult neural stem cells (aNSCs) maintenance and neural diseases remains unclear. To elucidate the function of miRNAs in aNSCs, we focused on DGCR8 gene, a possible candidate for 22q11-deletion type schizophrenia, and analyzed its function by generating knockout mice. As previously reported by Stark KL et al., DGCR8 knockout mice died before birth, but DGCR8(+/-) mice displayed schizophrenia-like phenotype with no obvious morphological changes in brain. To unveil the mechanism, we examined adult neurogenesis in hippocampal dentate gyrus. Interestingly, both aNSC proliferation and neurogenesis were significantly retarded in DGCR8(+/-) mice. When we further tested the self-renewal potential of aNSCs using neurosphere assay, neurosphere-forming ability was also down regulated in these mice. On the other hand, we could not observe any significant induction of active caspase-3-positive apoptotic cells in DGCR8(+/-) mice. Finally, we performed microarray-based differential gene expression profiling and found that several schizophrenia-related genes were down regulated in the hippocampus of DGCR8(+/-) mice. Among these genes, we found that gene X can restore proliferation of DGCR8(+/-) aNSCs both in vitro and *in vivo*. These results suggest that gene X contributes to the self-renewal potential of aNSCs in the hippocampal dentate gyrus and may play a critical role in development of schizophrenia in 22q11-deletion syndrome.

Poster Board Number: F-1060

FOXG1 IS REQUIRED IN LATE MOUSE NEOCORTICAL PROGENITORS TO DIRECT THE DIFFERENTIATION OF UPPER-LAYER PROJECTION NEURONS

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The mammalian neocortex comprises of diverse glutamatergic neuron populations, such as the preplate, deep-layer (layers V/VI) and upper-layer (layers II-IV) projection neurons. Each layer subtype arises from common neural progenitor cells (or neural stem cells) in a stereotypical temporal order. Previous in vitro and *in vivo* experiments have shown that the specification of deep-layer subtypes during the early corticogenesis period is largely regulated by intrinsic determinants within the progenitors. However, the mechanisms underlying the progression of progenitor cell competence resulting in a switch from deep- to upper-layer neuron production remain largely unknown. Foxg1 is a forkhead transcription factor that plays pleiotropic roles in the proliferation and differentiation of cortical progenitor cells. We previously identified that Foxg1 suppresses earliest glutamatergic cell identity within the deep-layer progenitors through the repression of multiple gene expressions. These results demonstrated that Foxg1 is a key regulator of temporal cell competence to switch to the generation of deep-layer projection neurons during the early corticogenesis period. In contrast, the requirement and specific roles of Foxg1 in late cortical progenitor cells has remained elusive. To directly assess the role of Foxg1 in upper-layer projection neuron development, we utilized a conditional knockout mouse line in which Foxg1 expression can be regulated under the control of tet-transactivator. Using this line, we inactivated Foxg1 at E14.5 by doxycycline administration and examined the development of upper-layer neurons. Analysis of these Foxg1 mutant cortices showed that the expression of upper-layer neuron specific genes was lost, and the migration of newly born neurons into the cortical plate were impaired. These data implied that Foxg1 is required in the late cortical progenitor cells to direct the differentiation of upper-layer projection neurons. Interestingly, temporal transcriptome analysis suggested that Foxg1 may regulate distinct molecular events within early and late cortical progenitor cells to control their neuronal differentiation. Collectively, these data imply that Foxg1 is a critical coordinator of neurogenesis program to direct layer-specific projection neuron differentiation throughout mammalian neocortical development.

Poster Board Number: F-1061

GENE EXPRESSION PROFILING ON DIFFERENTIATION OF MOUSE EMBRYONIC NEURAL STEM CELL EXPOSED TO ALCOHOL.

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Neural stem cells (NSCs) are tissue-specific multipotent stem cells that have the ability to differentiate into three cell lineages in the central nervous system: neurons, astrocytes and oligodendrocytes. The great therapeutic potential of NSCs has fueled attempts to characterize the expression of genes that regulate their fate. In this study, NSCs from the forebrain of embryonic day 15 mouse embryos were differentiated for 2 days in the presence or absence of ethanol (50 mM), and gene expression patterns between differentiated cells and ethanol-treated differentiated cells were assessed by microarray and real-time PCR analysis. Among the various analyzed genes, 336 genes were up-regulated in differentiated cells with presence of ethanol relative to differentiation cells with absence of ethanol whereas 1100 genes were down-regulated. Among the up-regulated genes, there were genes that were associated with an axon guidance protein (Ngef), p53 negative regulater (Letmd1) and DNA replication licensing factor (Mcm2). Among the down-regulated genes, there were genes that were associated with a glial marker (Gfap), a neuronal marker (Tubb3) and a glial transcription factor (Nfia). We found top three gene networks using GeneGo network analysis; TGF/WNT signaling pathway (Serping1, Wnt5a, and Csnk2a1), which is involved in specific gene transcription in brain development; cell cycle regulation of G1/S transition (Cdkn2a, Ccnd1, and Gsk3b); and IGF-1 receptor signaling pathway

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(Igf1r, Pten and Ywhaz), which is involved in cell proliferation and development. Most of genes included in top three gene networks were down-regulated in ethanol-treated differentiated cells compared to differentiated cells. Our results indicate that alcohol might negatively affect TGF/WNT and IGF-1 receptor signaling pathways during NSC differentiation.

Poster Board Number: F-1062

NO INDUCES DNA DAMAGE IN NEURAL STEM CELLS DERIVED FROM NIEMANN-PICK DESEASE TYPE C MICE

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Aims: Niemann-Pick disease type C (NPC) is typically associated with altered cholesterol metabolism and characterized by neurological deficits, eventually leading to premature death. Given the evidence that NO production in NPC inhibited self-renewal of neural stem cells (NSCs), we determined how NO expression disrupts neurogenesis and its involved pathological cellular signaling in NPC mice. Results: NPC-NSCs produced excessive NO and usually formed fewer, smaller neurospheres than WT-NSCs did. To confirm the role of NO, we treated NPC-NSCs with L-NAME, a NO synthase inhibitor and found that L-NAME rescue the impaired self-renewal of NPC-NSCs. Interestingly, we found that phosphorylated H2AX (γH2AX) foci, an indicator for DNA damage, were abundantly diffused in the NPC-NSC population, while the L-NAME treatment decreased the number of damaged cells. Since NO-induced DNA damage and apoptosis of NPC-NSCs also occur *in vivo*, we administered L-NAME to 4- week-old WT and NPC mice by i.p route for 2.5weeks to reduce the NO production. Importantly, L-NAME treated NPC mice contain approximately half of damaged cells of untreated NPC mice whereas no discernible change was found between WT groups, implying the pathological role of NO in NPC. Innovation and Conclusion: It remains to be established how NPC1 deficiency influences NSCs. In this study, we suggest NO as an important pathological factor in NPC etiology in the aspect of NSC damage. Moreover, given that treatment of L-NAME to NPC-NSCs prevents them from DNA damage, we propose NOS inhibitor as a possible therapeutic agent for NPC.

Poster Board Number: F-1063

DIRECT CONVERSION OF SOMATIC CELLS INTO NEURAL LINEAGES

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The reprogramming of mouse and human fibroblasts to induced pluripotent stem cells (iPSCs) raised the possibility that somatic cells could be reprogrammed to alternative cell fate without first becoming a pluripotent stem cell or a precursor cell. Recently, it was reported that mouse and human fibroblasts were directly reprogrammed to functional neurons using the combination of developmental transcription factors such as Ascl1, Brn2 and Myt1l. However, better cell type is needed for clinical applications because the fully differentiated neuronal cells are not good cell sources for regenerative medicine. In this study, we investigated whether mouse fibroblasts could be reprogrammed to progenitor cells, which will be a good cell type for cell transplantation, using Ascl1 and other neuronal factors. First of all, we established a protocol for direct conversion of the fibroblasts into neural lineages, such as neurons, astrocytes, oligodendrocytes and dopaminergic neuronal cells. Each cell type was analyzed with neural cell-specific markers such as nestin, sox1, tuj1, O4, GFAP, nurr1, lmxla and TH in our immunostaining and RT-PCR experiments. Interestingly, we found neural precursor cells during neural induction of the fibroblasts, which were positive against sox1 and nestin antibodies, and expressed neural precursor marker genes such as sox1, nestin, and others. Therefore, these results show that the mouse fibroblasts may be directly reprogrammed to neural lineages including neural precursor cells. However, it should be further studied whether the neural precursor cells could be directly differentiated into functional neurons in vitro and *in vivo*.

Poster Board Number: F-1064

PHENOTYPICALLY TRANFORMED GLIAL CELLS ACCUMULATE IN ADULT SPINAL CORDS OF TRANSGENIC RATS WITH MOTOR NEURON DEGENERATION.

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Background & Objective: Amyotrophic lateral sclerosis (ALS) is a progressive and fatal neurodegenerative disease characterized by adult-onset loss of motor neurons. A substantial neurogenesis in the degenerating spinal cord has not been reported in human ALS samples and also in rodent ALS models. Glial cells are believed to form non-permissive microenvironment for regeneration in the damaged adult spinal cord. Considering cell-based therapy in ALS, therefore, we focused on the phenotype of glial cells surrounding spinal motor neurons of a rat model ALS. Methods: We investigated the cellular phenotype of newly-generated glial cells that constitute the microenvironment in spinal ventral horns of His46Arg mutant SOD1 transgenic (Tg) rats at pre-symptomatic, early symptomatic, and late symptomatic stages (n=4-5) with their age-matched non-transgenic littermates (non-Tg). Continuous administration of bromodeoxyuridine for 7 days labelled newborn cells *in vivo*. After the administration, we performed multiple immunohistochemistry in lumbar spinal cord cryosections using various cell-selective markers for neural stem/progenitor cells, glial precursors, astrocytes, oligodendrocytes, and microglia. For quantification, we captured digital images of the immunofluorescence under confocal laser-scanning microscopy. Results: In contrast to non-Tg, the Tg rats showed a significant and progressive increase of newborn glial cells including glial precursors, astrocytes, and microglia. The gliogenic response in Tg rats was detected at the site of motor neuron degeneration from the early stage of disease. In the spinal ventral horns of symptomatic Tg rats, both the aberrantly activated astrocytes with immature phenotype and NG2-expressing phagocytic microglia accumulated progressively leading to severe neuroinflammation at the later stage. Conclusions: The present results revealed an emergence of phenotypically transformed glial cells that accumulate at the site of motor neuron degeneration in the ALS-like disease. Although the significance of the transformed glial cells remains to be addressed by intervention studies, coexistence of severe neuroinflammation suggests a non-permissive microenvironment in this ALS model. In addition to motor neuron restoration, glial control and prevention of neuroinflammation could be needed for promoting cell-based therapy in ALS.

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Poster Board Number: F-1065

EFFECTS OF REACTIVE OXYGEN SPECIES IN RAT ADULT NEURAL STEM/PROGENITOR CELLS DIFFERENTIATION

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Neural stem/progenitor cells (NPCs) persist in the adult organism and have been suggested to constitute a source for regeneration during inflammatory conditions such as central nervous system (CNS) injuries and Multiple Sclerosis. Oxidative stress (OS) has a very important role in the progress of inflammation. OS is mediated by reactive oxygen species (ROS), among which are hydrogen peroxide (H2O2), hydroxyl radical (HO) and superoxide (O2·[[Unsupported Character - ̄]]). ROS can cause lipid peroxidation, DNA damage and also neuronal cell death. The aim of this work is to dissect the effects of ROS, with special focus on H2O2, on the differentiation potential of NPCs. Primary NPC cultures were propagated from biopsies from the subventricular zone of adult Dark Agouti rats and were exposed to different concentrations of H2O2. Following the exposure, the H2O2 was washed away and the NPCs were differentiated for 5 days. The differentiation was assayed using immunocytochemistry for cell specific markers (gfap for astrocytes, tuj for neurons and O4 for oligodendrocytes) and quantitative RT-PCR (QPCR). H2O2 has a dual role, at high concentrations it can act as a free radical, and at low concentrations H2O2 serves as a signaling molecule. Our results show that at low H2O2 concentrations, exposed NPCs generated more neurons and oligodendrocytes following differentiation, compared to unexposed NPCs. During differentiation the cell exhibits different oxidative defense profiles. In order to investigate the mechanism by which the H2O2 effect is mediated, the expression of 84 OS-related genes was measured using QPCR. Three genes involved in the glutathione metabolism pathway; glutathione peroxidase 2 (GPX-2), glutathione peroxidase 6 (GPX-6) and glutathione peroxidase 4 (GPX-4) which play an important role in detoxifying the cell from ROS, had lower expression in cultures exposed to low H2O2 concentrations than in unexposed cultures. Lower oxidative defense in the NPCs could lead to a better penetrance of H2O2 to influence the differentiation result. In other cell types vascular endothelial growth factor A (vegfa) and its receptor vegfr1 expression have been demonstrated to be induced by ROS. Moreover, others have shown that vegf has a neurogenic effect on NPCs. We therefore decided to investigate whether the expression of vegfa and vegfr1 was affected by H2O2 in NPCs. Our results showed an upregulation of both vegfa and vegfr1 gene expression in NPCs exposed to low H2O2 concentrations compared to unexposed NPCs. This suggests a possible correlation with the observed neurogenesis at low H2O2 concentrations. In contrast, exposing NPCs to inflammatory levels of H2O2, resulted in lower astrocyte and neuron percentages in differentiated exposed cultures, compared to cultures from unexposed NPCs. Our results demonstrate that the differentiation process of adult NPCs can be influenced by environmental factors such as H2O2. The concentrations of the different ROS species could tune the differentiation event on NPCs, affecting the potential for the regeneration in the CNS.

Poster Board Number: F-1066

INTRAVENOUSLY ADMINISTERED AMNIOTIC FLUID-DERIVED NEURAL PROGENITOR CELLS INDUCE ENDOGENOUS CELL PROLIFERATION AND AMELIORATE BEHAVIORAL DEFICITS IN ISCHEMIC STROKE RATS

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Viable neural progenitor cells can be isolated from rat amniotic fluid (Antonucci et al., Cell Transplant., 2011). Here, we tested the therapeutic benefits of amniotic fluid-derived stem (AFS) cells in a rodent of model of ischemic stroke. Adult, male Sprague-Dawley rats (about 8 weeks old at study initiation and weighing ~250g) were initially trained in the cognitive task Morris water maze. Only those animals (n=16 from original 20 rats) reaching the learning criteria (e.g., ability to reach the hidden platform in less than 30 sec) were subsequently tested in motor tasks (elevated body swing test, rotorod test) and neurologic test, which further confirmed that these animal subjects were exhibiting normal behaviors (i.e., 50% swing activity to both left and right directions, ability to stay on rotating rod for at least 60 sec, and a neurologic mean score of close to zero based on a battery of somatosensory tests). These animals then received a one-hour occlusion of the middle cerebral artery followed by reperfusion, which was verified by laser Doppler and with routine physiologic parameters (e.g., blood gases) validating a homogenous stroke subject population. At 5 weeks post-stroke, animals that showed significant cognitive, motor, and neurologic deficits (n=14) subsequently received intravenous transplants of rat amniotic fluid-derived neural progenitor cells (1 million viable cells in 1 ml of sterile saline; n=7) or vehicle (equivalent volume of saline; n=7) delivered over a period of 1 minute. At about one month after transplantation, animals were subjected again to the same behavioral tests then euthanized for immunohistochemical evaluation of brain pathology. Statistical analyses revealed significant recovery of cognitive, motor and neurologic function in stroke animals that received the amniotic fluid-derived neural progenitor cells compared to vehicle-infused stroke animals (p´s< 0.05). Although the H&E staining revealed no significant differences in the infarcted core areas between the two stroke groups, the cell proliferation marker, Ki67, demonstrated at least a two-fold increase in Ki67-positive cells along the subventricular zone of the stroke animals that received amniotic fluid-derived neural progenitor cells compared to those that received vehicle infusion. Morevoer, there is also a corresponding increase in cells immunostained with Ki67 and doubled labeled with the migratory neural immature marker doublecortin. This increased cell proliferation along a neural fate occurred despite very few surviving grafts of amniotic fluid-derived neural progenitor cells. Parallel ELISA and gene microarray analyses revealed significant upregulation of the chemokine receptor for stromal cell-derived factor-1, CXCR4, and the vascular endothelial growth factor (VEGF), implicated in cell migration and cell proliferation, respectively. This study reports the therapeutic potential of amniotic fluid-derived neural progenitor cells in stroke animals, character-

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ized by attenuation of stroke-induced behavioral deficits, possibly via enhancement of endogenous repair mechanisms.

Poster Board Number: F-1067

3D DIFFERENTIATION OF PORCINE NEURAL PROGENITOR CELLS INTO SUBTYPE-SPECIFIC NEURONS AND GLIAL CELLS

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In vitro monolayer culture of mammalian neurons is a technique widely used in biomedical research to study developmental neurology and in drug discovery for screening of new disease-modifying drugs. However, neurophysiological and pathological properties are difficult to recapitulate in this artificial culture system. Hence, three dimensional (3D) culture systems have been developed to produce engineered neural tissues (ENTs), which are more suitable for the analyses of complex cell interactions. In this study, a porcine epiblast-derived neural progenitor cell (NPC) line (Rasmussen et al., 2011, Stem Cell Res 7:124-136) was used to produce ENTs. The NPCs were cultured for one week as neurospheres (NS) in NPC medium containing DMEM/F12, 1% N2, 1% B27, 1% Pen/strep, 20 ng/ml bFGF and 20 ng/ml EGF. The NS were then transferred to three (25 to each) hydrophilic membranes (BioCell, Switzerland) and 400 µl of differentiation medium containing DMEM/F12, 1% N2, and 1% Pen/strep, was added between the ENT inserts and the wells. After 2 days in 3D culture, the NS merged into a single structure and by Day 35, it had developed into a spherical 3D structure. At this point, the ENTs were fixed in 4% paraformaldehyde, cryosectioned and analyzed by immunohistochemistry (IHC) or fixed in 3% glutaraldehyde and processed for transmission electron microscopy (TEM). IHC of the sectioned ENTs showed widespread staining for the neural marker TUJI, the astrocyte marker GFAP, and the oligodendrocyte marker CNPase, underlining the multipotency of the NPCs. Neurons located within the upper part of the ENTs were positive for the mature neural marker NeuN, and staining for synaptophysin, a marker of presynaptic and neurosecretory vesicles, was also present in the upper layer and in the edges of the ENTs. The cholinergic marker ChAT, marked only a thin layer of cells in the uppermost part of the ENTs, whereas, staining with the glutamatergic marker VGLUT, revealed widespread staining, suggesting that most neurons had differentiated towards a glutamatergic subtype. Finally, the NPC marker NESTIN was negative, showing that all NPCs had undergone neural differentiation. TEM analyses showed that the ENTs were composed of densely packed cellular material with very sparse intercellular spaces, characteristic of neural tissue. Neuron-like cells presented a large spherical nucleus rich in euchromatin with a well-developed nucleolus, abundant rough endoplasmatic reticulum, mitochondria, intermediate filaments and axon-like structures presenting microtubules and vesicles located in bulbous endings. Such neuron-like cells were found in close contact with glial-like cells presenting lobulated nuclei rich in heterochromatin along the nuclear envelope. In conclusion, we have established a 3D culture system for generation of subtype-specific neurons and glial cells from porcine NPCs, which may potentially be used to capture advanced neural properties.

Poster Board Number: F-1068

DISTINCTIVE EFFECTS OF ARACHIDONIC ACID AND DOCOSAHEXAENOIC ACID ON RAT NEURAL STEM/PROGENITOR CELLS

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Arachidonic acid (ARA) and docosahexaenoic acid (DHA), which are the dominant polyunsaturated fatty acids in the brain, have crucial roles in brain development and function. We have previously shown that ARA promote postnatal neurogenesis in the rat hippocampus. In addition, another group has shown that DHA promote adult neurogenesis in the hippocampi of rats fed with a fish-oil deficient diet over three generations. However, the direct effects of ARA on neural stem/progenitor cells (NSPCs) and the effects of ARA and DHA on NSPCs at the neurogenic and subsequent gliogenic stages are still unknown. To further elucidate functions of these fatty acids on NSPCs, we analyzed effects of ARA and DHA on the maintenance and differentiation of NSPCs using in vitro neurosphere assays. Here, we used primary neurospheres as neurogenic NSPCs and tertiary neurospheres as gliogenic NSPCs. To examine the effects on maintenance of NSPCs, neurospheres were dissociated and incubated with various concentrations of ARA or DHA. After 7 days in culture, the number of neurospheres was counted. To examine the effects on differentiation of NSPCs, neurospheres were dissociated and cultured with various concentrations of ARA or DHA. After 4 days in culture, the number of neurons or astrocytes was counted. Regarding the effects of ARA and DHA on neurogenic NSPCs, ARA at 10-6 M increased the number of neurospheres and so did DHA at 10-7 M, whereas neither ARA nor DHA had a detectable effect on the differentiation of NSPCs. As for the effects of ARA and DHA on gliogenic NSPCs, DHA at 10-10 M and 10-8 M increased the number of neurospheres, while ARA had no such effect. On the other hand, ARA at 10-5 M increased the number of astrocytes, whereas DHA at 10-7 M increased that of neurons. These data suggest that ARA promotes the maintenance of neurogenic NSPCs and induces the glial differentiation of gliogenic NSPCs and that DHA promotes the maintenance of both neurogenic and gliogenic NSPCs and induces the neuronal differentiaiton of gliogenic NSPCs. We are now investigating molecular mechanisms of ARA and DHA on the maintenance and differentiation of NSPCs and the effects of these fatty acids on NSPCs *in vivo*.

Poster Board Number: F-1069

THE CHARACTERIZATION OF BASAL RADIAL GLIA IN THE DEVELOPING MATMOSET BRAIN

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The progenitors in the subventricular zone (SVZ) significantly contribute to the neocortex development. A recent study revealed a novel type of SVZ progenitor, referred to as basal radial glia (bRG), which retains a basal process to the pial surface, sustains expression of radial glial markers and is capable of self-renewal. bRGs occur at high relative abundance in the outer SVZ (OSVZ) of gyrencephalic primates (human) and nonprimates (ferret), but lower of lissencephalic rodents (mouse), suggesting that the division of bRG in OSVZ is important to generate gyrencephalic brain. The common marmoset, Callithrix jacchus, is a near-lissencephalic primate despite the similar abundance of bRG cells to that in human and

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ferret neocortex. Our recent paper (Kelava et al, 2011) revealed that a frequent occurrence of bRG cells is not sufficient to develop gyrencephaly and suggests that multiple parameters, such as progenitor cell type and cell cycle kinetics, may determine whether the neocortex become lissencephalic or gyrencephalic. In this study, we sought to explore the timing of bRG emergence in marmoset OSVZ as well as the fate of bRG cells. We characterized bRG cells in developing marmoset OSVZ using the technique of sequential 5′ -bromo-2′ -deoxyuridine (BrdU) and 5-ethynyl-2′ -deoxyuridine (EdU) labeling by intraperitoneal injection of BrdU and EdU to pregnant mother, in combination with immunofluorescent staining of brain slice. Our observations demonstrated that the RG-like cells in OSVZ first appeared at E86 to E89. We also analyzed the cell cycle length of marmoset neocortical progenitors by counting the EdU+/BrdU+, EdU+/BrdU- and EdU-/BrdU+ RG-like cells. In addition, we finally performed live imaging of bRG using a cortical slice culture technique and observed the birth of bRG cells, their division pattern in OSVZ and the fate of their daughter cells.

Poster Board Number: F-1070

NEURAL STEM CELLS, ADULT NEUROGENESIS AND GALECTIN-1: FROM BENCH TO BEDSIDE

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Neural stem cells (NSCs) in the adult brain have been a consistent focus of biomedical research. One reason is because of their potential clinical applications. To fully exploit this potential, the molecular mechanisms that regulate NSCs must be clarified. Several lines of evidence show that a multi-functional protein, Galectin-1, is expressed and has a functional role in a subset of adult NSCs. Researchers, including our group, have explored the physiological role of Galectin-1 in NSCs and its application in the treatment of animal models of neurological disorders such as brain ischemia and spinal cord injury. Here, we summarize what is currently known regarding the role of Galecitin-1 in adult NSCs. Furthermore, we discuss current issues in researching the role of Galectin-1 in adult NSCs under both physiological and pathological conditions.

Poster Board Number: F-1071

DIFFERENTIAL EFFECTS OF PRENATAL VS ADULT INFLAMMATORY STIMULI ON ADULT NEUROGENESIS AND THE NEUROGENIC NICHE.

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The perinatal stage is characterized by increased sensitivity to inflammatory stimuli which affect adult physiology, including adult neurogenesis. We have hypothesized that a prenatal pro-inflammatory challenge would cause a more sustained negative effect on adult neurogenesis than an adult one. To test this hypothesis, we injected Wistar pregnant rats subcutaneously with lipopolysaccharide (LPS, 0.5 mg/kg) or saline at embryonic days 14, 16, 18 and 20. In the adulthood, a similar regime (4 subcutaneous LPS injections every other day, 1 mg/kg) or a single ip. LPS dose (1 mg/kg) were administered. The effects on neurogenesis were assessed 60 days later in both cases. We have observed 25% reduction of adult neurogenesis levels and an impaired performance in the novel object recognition test in prenatally LPS-treated rats compared to controls. Both effects were mediated by a decrease in the levels of TGF-beta and accompanied by a long-term microglial activation in the dentate gyrus. In contrast, we found that adult LPS treatment significantly decreased adult neurogenesis after 7 days but not after 60 days, assessed by co-labeling of BrdU and neuronal markers (PSA-NCAM; DCX). In line with these results, microglial activation in adult LPS treated animals was only observed in correlation with decreased neurogenesis. Even though, cytokine expression analysis by real-time RT-PCR revealed a decrease in TGF-beta expression in the adult hippocampi of prenatally LPS-treated rats, the levels of TGF-beta expression in the DG of the adult treated animals remained unchanged. Hence, peripheral inflammation has different outcomes regarding adult neurogenesis and the neurogenic niche, according to the developmental stage when the challenge is received. These results highlight the susceptibility of the CNS to prenatal programming and its long term consequences compared to the limited response of the adult brain.

Poster Board Number: F-1072

NEURAL STEM CELL GRAFTING COUNTERACTS HIPPOCAMPAL-INJURY MEDIATED IMPAIRMENTS IN MOOD, MEMORY AND NEUROGENESIS

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Hippocampus is vital for functions such as mood and memory. Hippocampal injury typically leads to mood and memory impairments associated with reduced and aberrant neurogenesis in the dentate gyrus. In this context, interventions that are competent for averting the evolution of initial hippocampal injury into mood and memory impairments have great significance. Particularly, therapeutic strategies that have promise for maintaining neurogenesis in the injured hippocampus at normal levels in terms of both extent and pattern are of great interest. This is because hippocampal neurogenesis is considered to be vital for functions such as mood and memory, and the aberrant neurogenesis that ensues after injury is believed to contribute to mood and memory dysfunction as well as dentate hyperexcitability. We examined whether neural stem cell (NSC) grafting after hippocampal injury would counteract impairments in mood, memory and neurogenesis. We expanded NSCs from the anterior subventricular zone (SVZ) of postnatal F344 rat pups expressing the human placental alkaline phosphatase and grafted into the hippocampus of young adult F344 rats at five days after an injury inflicted through a unilateral intracerebroventricular administration of kainic acid. We chose postnatal SVZ-derived NSCs as donor cells in this study because of the feasibility for their expansion in culture for extended periods without losing multipotency owing to their self-renewal ability. Moreover, harvesting of SVZ-NSCs from autopsied postnatal or adult human brains is also feasible. Analyses through forced swim, water maze, and novel object recognition tests revealed significant impairments in mood and memory function in animals that underwent injury and sham-grafting surgery. In contrast, animals that received SVZ-NSC grafts after injury exhibited mood and memory function that are comparable to naïve control animals. Graft-derived cells exhibited excellent survival and pervasive migration, and differentiated into neurons, subtypes of inhibitory GABA-ergic interneurons, astrocytes, oligodendrocytes and oligodendrocyte progenitors. Significant fractions of graft-derived cells also expressed beneficial neurotrophic factors such as the glial cell-line derived neurotrophic factor, brain-derived neurotrophic factor and fibroblast growth factor. Furthermore, SVZ-NSC grafting counteracted the injury induced reductions and abnormalities in neurogenesis by both maintaining a normal level of NSC activity in the subgranular zone and providing protection to reelin+ interneurons in the dentate gyrus. These results underscore

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that early SVZ-NSC grafting intervention after hippocampal injury is efficacious for thwarting mood and memory dysfunction and abnormal neurogenesis.

Poster Board Number: F-1073

GLP1R-SIRT1 CASCADE IN HESC AND IPSCS REGULATING PLURIPOTENCY AND DIFFERENTIATION

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The glucoinectrin hormone glucagon-like peptide 1-[7,36]amide (GLP-1) exerts several biological effects on peripheral tissues and the central nervous system (CNS). Recently we have demonstrated the presence of GLP-1 receptors in human embryonic stem cells (hESCs) and induced pluripotent stem (iPS) cells. Our preliminary data on micro RNA indicate that these receptors may have a role in maintaining pluripotency in these cells. However, the molecular mechanism by which GLP-1 receptors are involved in this process is not fully elucidated. Sirtuins or 'silent information regulators of gene transcription' are a new class of NAD^+-dependent histone deacetylases that have been linked to a longer lifespan. While seven mammalian sirtuins have been identified, numerous studies suggest that SIRT1 is involved in neuropathology, although the role of this protein in normal cognitive functions is not clear. In this study, we aimed to determine whether GLP-1R ligand interactions act through sirtuins in influencing proliferation and apoptosis of hESCs and iPS derived neuronal stem cells. To address these issues, we tested mRNA levels of human SIRT1-7, and their associated protein in human embryonic stem cells (hESCs), induced pluropotent stem cells (iPS cells) derived from Alzheimer's disease patients and age-matched controls. We have reported the presence of SIRT1-7 in all hESCs and iPS cells using quantitative PCR. Our data shows that GLP-1 receptor expression increases with pluripotency and differentiation in hESCs occurring concurrently with a decrease in SIRT1 and SIRT2 mRNA expression. Contrary to the SIRT1-2 data, a significant increase in SIRT3-7 mRNA expression was also observed during hESC propagation. This preliminary data indicates that the GLP-1R-sirtuin interactions are crucial for maintaining dopaminergic neurons derived from pluripotent stem cells.

Poster Board Number: F-1074

MECHANORECEPTIVE NEURAL NETWORK WITH RNA BINDING PROTEIN MUSASHI2

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The Musashi (Msi) proteins comprise a family of highly conserved RNA-binding proteins that regulate the expression of target proteins at the translational level. *Drosophila*-Msi regulates asymmetric cell division in neural development. In mammalian central nervous system (CNS), Msi1 and Msi2, are co-expressed in neural stem cells, suggesting Msi family proteins maintain the mouse stem cells in CNS. To reveal the roles of Msi in vivo, we generated msi1 deficient mice which frequently died of obstructive hydrocephalus. The result from msi1 deficient mice also indicated that the Msi2 have cooperative roles, thus we generated msi2 deficient mice. The msi2 deficient mice survived to adult showing the phenotype of hypersensitivity to touch (allodynia) and fewer-than-normal synapses in the deep layer of the dorsal horn of the spinal cord with fewer innervation of sensory axons from the dorsal root ganglion (DRG) to the appropriate target layer in spinal cord. Target mRNA screening of Msi2 revealed that one of the target mRNAs control the neurite out growth. The direct regulation of the target by Msi2 was confirmed with reporter and gel shift assay. Detailed behavioral and histological analyses confirmed that the Msi2 regulate the projection of specific timing and specific subtypes of sensory neurons by controlling the expression of the target. The phenotypes of msi2 deficient mice indicate that Msi2 regulates the formation of the sensory neural network by controlling protein expression of its target.

Poster Board Number: F-1075

SUPPRESSION OF TUMOR FORMATION IN STEM CELL THERAPY FOR PARKINSON'S DISEASE

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Loss of dopaminergic neurons is a hallmark of Parkinson's disease (PD). Embryonic stem cell techniques focusing on restoration of this single cell type have the potential to work as treatment of PD. We have conducted a drug screen aiming for tumor suppression associated with ES cell therapy. An anionic drug (AD) was selected within a screen where 24 drugs had been evaluated. The selection criteria included known, approved drugs, negatively charged and with small MW (<350 Da), known to promote differentiation or inhibit proliferation. Mouse ESCs (E 14.1 line) were pre-differentiated for 5 days and subsequently treated for 24 hours with a high dose of the anionic drug and cells were analysed at day 21 of differentiation for the expression of the markers, Nestin, TH, Tuj1 and Nurr1 by immunostaining, real time QPCR and FACS. The results indicate that the expression of tuj, TH and Nurr1 increase on samples treated with the AD and that, most importantly, the expression of Nurr1 and TH relative to nestin in mESCs treated with the anionic drug is about 2 times higher than that in the untreated cultures suggesting that the drug selected is promising for dopaminergic differentiation and suppression of proliferation.

Poster Board Number: F-1076

PAIRED RELATED HOMEOBOX PROTEIN 1 IS A NOVEL KEY REGULATOR OF ADULT NEURAL STEM/PROGENITOR CELLS

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Newborn neurons are generated from neural stem cells (NSCs) in two specific regions of the adult brain. Maintenance for the self-renewal activity and multipotency of adult NSCs are controlled by multiple transcription factor networks. We show here that paired related homeobox protein Prx1 (MHox1/PRRX1) plays an important role in the maintenance of adult NSCs. Prx1 protein is expressed in Sox2+/GFAP+/Nestin+ astrocytes in the subventricular zone of the lateral ventricles and the subgranular zone of the dentate gyrus in the adult brain, and work with the transcription factor Sox2 as a co-activator. In addition, depletion of Prx1 in cultured adult NSCs lose their self-renewal activity. Furthermore, forced expression of Prx1 in the proliferating adult hippocampal stem/progenitor (AHP) progeny lead to the exclusive generation of type-I/IIa-like astrocytes and oligodendrocytic progenitor-like lineages at the expense of newborn neurons. These data suggest that Prx1 plays an important role in neural cell lineage determination, and maintenance for the self-renewal of adult NSCs at several stages in the adult brain.

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Poster Board Number: F-1077

IPS CELL-DERIVED NEURAL CREST CELLS FROM A PATIENT WITH HIRSCHSPRUNG'S DISEASE SHOW A REDUCED DIFFERENTIATION PLASTICITY

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Hirschsprung's disease (HSCR) is a congenital disease characterized by the absence of ganglian cells in the colon. Some syndromic HSCR patients also present with cardiac defects, such as ventricular and atrial septal defects (VSD/ASD). These diseases would be attributed to the defects in differentiation and/or migration of enteric and cardiac neural crest (NC) cells. For a better understanding of disease pathogenesis of HSCR and the associated syndromes, our laboratory has recently established two iPS cell lines from a HSCR patient exhibiting a short segment aganglionosis and ASD/VSD. With a gradient switch from KSR medium to a neural inductive N2 medium supplemented with various neurotropic factors, human iPS cells differentiate to NC cells ($HNK1^+/p75^+$). Both the HSCR and control (IMR90) iPS lines could generate NC cells of similar capacity. However, we found that HSCR iPS cells exhibit a lower efficiency to produce enteric NC cells ($HNK1^+/RET^+$). A significantly less number of $HNK1^+/RET^+$ cells were obtained from HSCR iPS cells on day 9 in the differentiation medium. Despite the patient NC cells could differentiate further along neuronal lineage, number of neuronal precursors ($TH^+/Tuj1^+$) obtained from the patient lines was significantly reduced. In addition, they were not able to fully differentiate to mature neurons ($PGP9.5^+$) of proper neurite outgrowth and showed a reduced neural plasticity to form enteric neurons (such as VIP^+ neurons). Similarly, these patient iPS cell derived NC cells are less competent to form smooth muscle cells (SMA+) when compared to that of the control line. In summary, we have demonstrated that HSCR-iPS cell derived NC cells may harbor the intrinsic differentiation defects towards to neurogenic and smooth muscle cell lineages, which may lead to the incomplete colonization of the bowel and cardiac malformation of the patient.

Poster Board Number: F-1078

CALCIUM PHYSIOLOGY OF B TYPE ADULT RADIAL GLIA-LIKE PRECURSOR CELLS

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Adult neurogenesis is well established in the subventricular zone (SVZ) lining the lateral ventricles of the adult mammalian brain. Neural precursor cells of this neurogeneic niche continuously produce neurons that migrate, differentiation and become integrated into functional networks involved in odor discrimination in the olfactory bulb. Adult SVZ derived neurospheres are thought to contain multipotent neural stem cells and when plated *in vitro* were observed to generate cells of the three main neural lineages: βIIItubulin+ neurons (0.012 ± 0.057%); GFAP+/vimentin- astrocytes (0.033 ± 0.017%); and O4+ oligodendrocyte precursor cells (0.091 ± 0.13%). Calcium signalling was investigated in two neural phenotypes observed to emerge from neurospheres and identified as vimentin+/SOX2+/DCX-/GFAP- radial glia-like (B type) cells and DCX+/SOX2+/vimentin-/GFAP- neuronal precursor (A type) cells. Responses to the purinergic agonist ATP (50μM) and growth factor mitogens EGF (10ng/ml) and bFGF (10ng/ml) were examined and their downstream signalling cascades analyzed. Both B type and A type cells were found to be responsive to depolarization and exhibited mechanisms consistent with SOC channel activation. This study found that the spontaneous calcium oscillations observed in the B type, radial glia-like cells, was dependent on EGF but not on FGF. Through the use of the antagonists ruthenium red (10μM), ryanodine (100μM), 2-APB (5μM), xestospongin-C (1μM), U-73122 (5μM) and thapsigargin (1μM) spontaneous calcium oscillations were found to be regulated at the IP3R on the ER. Taken as a whole, these results offer a novel insight into the calcium physiology of SVZ-derived neural precursor cells.

Poster Board Number: F-1079

CHARACTERIZATION OF IPSC-DERIVED NEURONS FROM FAMILIAL ALZHEIMER'S DISEASE PATIENTS

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Alzheimer's disease (AD) is the most common cause of dementia and currently affects more than 26 million people worldwide. The majority of AD is sporadic without a known genetic or environmental cause. However, there is a small minority of patients with dominantly inherited forms of the disease caused by mutations in amyloid precursor protein (APP), Presenilin 1 (PS1) and Presenilin 2 (PS2). With the advent of induced pluripotent stem cell (iPSC) technology, we are now able to capture the genomes of these individuals and differentiate iPSCs into neurons and glia to investigate the molecular mechanisms that cause AD. The predominant hypothesis in the AD field implicates toxic amyloid-beta (Aβ) peptides in driving neuronal phenotypes. This hypothesis largely stems from the knowledge that the genes affected in fAD patients are all involved in the processing of APP and the generation of its secreted peptide, Aβ. Studies in the field however, suggest that neuronal autonomous events could also be taking part. We recently reported that in iPSC-derived neurons of APP duplication patients we see elevation of secreted Aβ, hyperphosphorylated tau, and GSK3β as well as enlarged Rab5-positive early endosomes (Israel et al. Nature, 2012). Here we extend our group's work to include the characterization of iPSC-derived neurons from patients with the APP V717F mutation. Importantly, IPSC-derived neurons from V717F patients display increased Aβ42 to 40 ratio, as expected, but not elevation of phosphorylated tau. This is in contrast to our group's findings in APP duplication patients and suggests that there are different molecular mechanisms driving the pathology of fAD even when the same gene, APP, is affected.

Poster Board Number: F-1080

INHIBITION OF CSPG PRODUCTION IN ASTROCYTE BY GM-CSF ENHANCES NEURITE OUTGROWTH OVER PC12 CELLS *IN VITRO*

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Granulocyte macrophage colony stimulating factor (GM-CSF) is a potent hematopoietic cytokine, which stimulates stem cell proliferation in the bone marrow and inhibits apoptotic cell death in various cells. Recently, GM-CSF is being investigated to play a role in central nervous system (CNS). In our previous studies, GM-CSF has been shown to inhibit the apoptosis of neural cells and glial scar formation in rat SCI model. Chondroitin sulfate proteoglycans (CSPGs) have been shown to be up regulated in the CNS following injury and to inhibit regeneration in vitro and *in vivo*. In response to scar-inducing factors, astrocytes up regulate the production of CSPGs. In this study, we investigated the effect of GM-CSF on the expression of CSPG core proteins and neurite outgrowth in vitro system. GM-CSF inhibited the expression of CSPGs such as

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neurocan, phosphacan and NG2 in rat astrocytes when treated with TGF-β3 or EGF. In addition, the neurite outgrowth of PC12 cells was enhanced when the cells were cultured on the extra-cellular matrix (ECM) over astrocyte treat with GM-CSF. These results provide the basis for the therapeutic effect of GM-CSF on neural cell injuries and for further expanding its therapeutic applications.

Poster Board Number: F-1081

SINGLE CELL ANALYSIS SHOWS MOSAIC CNVS IN HIPSC-DERIVED NEURONS AND FIBROBLASTS

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Diversity among individual cells is obscured by population-level bulk measurement. Single cell resolution is especially relevant in the nervous system, where several lines of evidence indicate extensive neuron-to-neuron genetic diversity (i.e. somatic mosaicism) in the brain. Given the unique access to human neurons provided through somatic cell reprogramming, we applied single cell genomic approaches to study neurons derived from karyotypically normal human induced pluripotent stem cell (hiPSC) lines. This work established that many of these neuronal genomes are variants of the individual's genome. In addition to aneuploid neurons, many large copy number variations [CNVs (i.e. sub-chromosomal amplifications and deletions)] were prevalent in euploid neurons. The paucity of common CNVs among hiPSC-derived neurons is consistent with their occurrence during or after cell cycle exit. These single cell genomic approaches were also applied to karyotypically normal cultured human fibroblasts. Large amplifications were much more prevalent in fibroblasts than in neurons. Most importantly, some large amplifications were shared by other fibroblasts, and could be confirmed by FISH in interphase nuclei from the karyotypically normal preparation. The prevalence of fibroblasts with mosaic genomes may contribute to the success of cellular reprogramming approaches.

Poster Board Number: F-1082

ACTIVIN TYPE I RECEPTOR (ALK4) IS A SWITCH FOR THE DUAL ACTION OF THE ACTIVIN/ALK4 PATHWAY: PROMOTING MESODERMAL LINEAGE AND INHIBITING NEURAL FATE

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During embryogenesis, the Activin/Nodal pathway plays a crucial role in lineage decision. *In vivo* studies showed that different concentrations of Activin elicit distinct responses from Xenopus animal caps, ultimately producing a range of mesodermal fates. Nodal-/- mice fail to form both the mesoderm and the definitive endoderm, but show precocious neural differentiation, suggesting that the Activin/Nodal pathway promotes mesodermal lineage and inhibits neural fate decision. This is corroborated by a series of studies in vitro. Blockage of Activin signalling has been shown to promote neural fate in human ES cells and in mouse ES cells (our unpublished data). What molecular mechanism underlies the dual role of the Activn/Nodal pathway is not clear. We have discovered that protein tyrosine phosphatase 1B (Ptp1B) acts as a novel partner of the Activin/Alk4 pathway to select between mesodermal or neural fates. We employed human and mouse embryonic stem (ES) cell-differentiation model to investigate the function of the Activin/Alk4 pathway in the early fate decision. We found that the treatment of Activin at the different stages of ES cell differentiation exhibit diverse influences in cell-type derivatives. Inhibition of the Activin pathway at different time windows also shows a stage-dependent fate adaptation. A downstream factor of this pathway, Ptp1B, has been identified to interact with Alk4, which in turn governs Activin-directed fate decision in combination with p-Smad2/3 signalling.

Poster Board Number: F-1083

HEPARAN SULFATE CONNECTIVE TISSUE NICHE FOR THE REGULATION OF STEM CELL PROLIFERATION IN THE ADULT BRAIN

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In adulthood, new neurons and glial cells are generated from stem cells in restricted zones of the brain, namely the olfactory bulb (OB), rostral migratory stream (RMS), subventricular zone (SVZ) of the lateral ventricle, sub-callosum zone (SCZ) and sub-granular layer (SGL) of the dentate gyrus. What makes these zones germinal? We previously reported that N-sulfated heparan sulfates (N-sulfated HS) present in specialized extracellular matrix structures (fractones) and vascular basement membranes bind and promote the mitotic and anti-mitotic activity of the neurogenic factors FGF-2 and BMP-7 respectively, next to stem cells in the anterior SVZ of the lateral ventricle, the most neurogenic zone in adulthood. Basement membranes-associated heparan sulfates are known to bind, store and protect bound growth factors from enzymatic degradation. Heparan sulfates are also known to facilitate the presentation of growth factors to their cognate receptors at the surface of target cells to ultimately mediate growth factor biological activity. Therefore, heparan sulfates in fractones and vascular basement membranes most likely promote the effect of FGF-2 and BMP-7 on mitosis to locally regulate neurogenesis in the adult neural stem cell niche. To determine to which extent cell proliferation is associated with N-sulfated HS, we mapped N-sulfated HS and proliferating cells by immunohistochemistry throughout the adult mouse brain. We found that cell proliferation is associated with N-sulfated HS in the OB, RMS, the whole germinal SVZ, and the SCZ. Cell proliferation was weakly associated with N-sulfated HS in the SGL, but the SGL was directly connected to a sub-cortical N-sulfated HS+ extension of the meninges. The NS-sulfated HS+ structures were blood vessels in the OB, RMS and SCZ, and primarily fractones in the SVZ. N-sulfated HS+ fractones, blood vessels and meninges formed a continuum that coursed along the OB, SVZ, RMS, SCZ and SGL, challenging the view that these structures are independent germinal entities. These results support the possibility that a single anatomical system might be globally responsible for mitogenesis and ultimately the production of new neurons and glial cells in the adult brain. Supported by NIH R21 NS057675, NIH RCMI 5G12/A103061 and Japanese Society for the Promotion of Scienes S09109.

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Poster Board Number: F-1084

DIRECT TRANSDIFFERENTIATION OF FIBROBLAST CELLS INTO TRIPOTENT NEURAL STEM CELLS THAT ARE CLONALLY EXPANDABLE AND EXHIBIT TRANSGENE SILENCING

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Transcription factor-driven reprogramming of fibroblast cells has been shown to yield neurons, cardiomyocytes, and neural as well as hepatocyte progenitors, demonstrating that transdifferentiation of somatic cells will develop into a new paradigm in regenerative medicine. While somatic stem cells can be derived either from primary tissue or pluripotent cell sources such as induced pluripotent stem (iPS) cells the artificial induction of somatic cells has remained elusive. Here we aim at the direct derivation of neural stem (NS) cells from fibroblasts employing a modified iPS reprogramming paradigm. We used retroviral transduction of Sox2, Klf4, c-Myc and Oct4 to initiate dedifferentiation of fibroblast cells employing neurogenic conditions. 19 days post infection we observed neurosphere-like colonies that could be readily isolated and clonally expanded both as sphere and adherent cultures. These cells uniformly exhibit morphological and molecular features of NS cells such as the expression of Nestin, Olig2, and Pax6. Moreover, data will be presented demonstrating they iNS cells are able to differentiate into all three neural lineages, neurons, astrocytes as well as oligodendrocytes in vitro and *in vivo*. Fibroblast-derived iNS cells exhibit clonal growth and maintain their marker expression profile and differentiation capability over prolonged expansion (>50 passages). We expect transdifferentiated somatic stem cells such as NS cells to provide a safe and robust, virtually unlimited source of patient-specific cells for future applications in regenerative medicine and basic research.

Poster Board Number: F-1085

NOVEL NEURAL STEM CELL MARKER, SUR8/SHOC2 INVOLVE BOTH INHIBITION OF DIFFERENTIATION AND MAINTENANCE OF STEMNESS OF NEURAL STEM CELL VIA ERK SIGNALING

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Sur8/Shoc2 is a scaffold protein that regulates the Ras-ERK pathway. However, the roles of Sur8 in cellular physiologies are poorly understood. In the present study, Sur8 was severely repressed in the course of neural stem cells (NSCs) differentiation in the cerebral cortex of developing rat embryos. Similarly, Sur8 was also critically reduced in cultured NSCs which were induced differentiation by removal of basic fibroblast growth factor (bFGF). Sur8 regulation occurs at the protein level rather than at the mRNA level as revealed by both in situ hybridization and RT-PCR analyses. The role of Sur8 in NSCs differentiation was confirmed by lentivirus-mediated Sur8 knock-down, which resulted in increased differentiation, while exogenous expression of Sur8 inhibited differentiation. Contrastingly, NSC proliferation was promoted by overexpression, but was suppressed by Sur8 knock-down. The role of Sur8 as an anti-differentiation factor in the developing rat brain was confirmed by an *ex vivo* embryo culture system combined with the lentivirus-mediated Sur8 knock-down. The numbers and sizes of neurospheres were reduced, but neuronal outgrowth was enhanced by the Sur8 knock-down. The Ras-ERK pathway is involved in Sur8-mediated regulations of differentiation, as the treatment of MEK inhibitors blocks the effects of Sur8. The regulations of $NSCs_i^-$ differentiation and proliferation by the Ras-ERK pathway were also shown by the rescues of the effects of bFGF depletion, neuronal differentiation and anti-proliferation by epidermal growth factor (EGF). In summary, Sur8 is an anti-differentiation factor that stimulates proliferation for maintenance of stemness in NSCs via modulation of the Ras-ERK pathway.

Poster Board Number: F-1086

ROLE OF NFIA TRANSCRIPTION FACTOR IN GLIOGENESIS

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In the developing CNS, the all types of neural cells are derived from multipotent neural stem cells (NSCs). However, since the differentiation potential of NSCs is spatiotemporally regulated during development, they cannot always generate all the neural cell types. For instance, NSCs in early gestation cannot differentiate into astrocyte because they have no competency to respond gliogenic differentiation signals yet. Thus, neurons come first then glia in developing brain. Despite of extensive analysis of cell fate determination processes of NSCs, the molecular mechanisms of temporal specification of NSCs is poorly understood. Nuclear Factor I is a family of closely related CCAAT box element-binding transcription factors composed of A, B, C and X in mammals. NFIA has been shown to be a key regulatory factor for NSCs to start gliogenesis. However, other than glial fibrillary acidic protein (GFAP), a marker of astrocyte, no key functional downstream effector of NFIA for the onset of gliogenesis has been identified. To identify the downstream factors of NFIA, we took an advantage of our original *in vitro* culture system using mouse embryonic stem cells (ESCs), in which NSCs are differentiated from ESCs through embryoid body (EB) formation and are selectively amplified as neurospheres. In this system, the temporal specification of NSCs is recapitulated and thus primary neurosphere from EBs generate only neurons, then gliogenesis is activated in subsequent generations of neurospheres. To confirm previous studies regarding the function of NFIA for gliogenesis, we first overexpressed NFIA in ESC- derived neurospheres via lentivirus vectors. Surprisingly, NFIA did not significantly facilitate gliogenesis in primary neurospheres which are highly neurogenic but did so in secondary neurospheres which are starting gliogenesis, indicating that some competence change is required for the gliogenic action of NFIA. Moreover, NFIA seemed to promote astrogliogenesis only if its overexpressing cells are localized with a mosaic-like pattern within ESCs-derived secondary neurospheres. To obtain the mosaic-like localization of the NFIA-overexpressing cells within neurospheres constantly, we established ESC lines that express NFIA in a doxycycline-dependent manner so-called Tet-off system and co-cultured NSCs derived from the Tet-off ESCs line and wild-type ESCs to form aggregated and mixed (WT/NFIA-mix) neurospheres. We confirmed that NFIA-overexpressing NSCs derived from WT/NFIA-mix spheres showed significant increase of astrocytic differentiation compared to neurospheres composed of only NFIA-overexpressing

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cells. This result suggests that the cell-cell interaction between progenitor cells with different levels of NFIA induces a lateral activation of astrocytic differentiation of progenitor cells with high NFIA expression. Interestingly, this action of NFIA was cancelled by a γ-secretase inhibitor (DAPT), suggesting a possible involvement of Notch signaling in this process. Finally, to identify downstream effectors of NFIA in gliogenesis, we isolated NFIA-overexpressing cells from the WT/NFIA-mix spheres from ESCs, then analysed global gene expression patterns by DNA-microarrray. We are currently performing functional screening of candidate genes and will investigate the relationship between NFIA and Notch signaling to reveal the molecular mechanisms underlying NFIA mediated gliogenesis by neural stem cells in the CNS development.

Poster Board Number: F-1087

ANALYSIS OF GLAST SORTED CELLS DERIVED FROM ES/IPS CELLS FOR APPLICATION OF CELL THERAPY

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Parkinson's disease (PD) is one of the neurodegenerative diseases and is characterized by the death of dopaminergic neurons in the substantia nigra. When selective degeneration of nigrostriatal dopaminergic neurons occurs, the symptoms of PD emerge. The main symptoms of motor abnormalities in PD are bradykinesia, resting tremor, rigidity, and postural instability. Current therapeutic approaches for PD such as pharmacological tools and deep brain stimulation provide symptomatic relief, but they cannot stop the progression of the disease. The prominent therapy for PD is a cell replacement. Studies in patients with PD after intrastriatal transplantation of human fetal mesencephalic tissue that includes many dopaminergic neurons have provided proof of principle that neuronal replacement can work in the human brain. However, it is difficult to prepare sufficient fetal brain tissue to transplant for one PD patient. Instead of fetal tissue, embryonic stem (ES) cells and induced pluripotent stem (iPS) cells are promising cell sources for cell-replacement therapy of PD. The glutamate transporter Glast is localized on the cell membrane of mature astrocytes and is also expressed in the ventricular zone of developing brains, a transient zone that produces most neurons and neuroglia. Recently, it was reported that dopaminergic neurons are generated by Glast-expressing radial glia-like progenitors in the floor plate. Therefore, Glast-expressing cells are available to sort neurons and astrocytes from differentiating ES/iPS cells. In this research, we used Glast as a cell surface marker, and characteristics of Glast-sorted cells derived from mouse ES/iPS cells were analyzed.

Poster Board Number: F-1088

OBSERVATION OF STEM CELL DYNAMICS AND IMPLICATION FOR HOW TO BUILD THE LARGER BRAIN.

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Our knowledge of the cellular basis of human neocortical development and the spatial organization of the developing primate neocortex has recently expanded considerably due to the recent discoveries concerning the cellular architecture and patterns of neurogenesis of the developing human brain, which will change the concepts of our understanding of human neurodevelopmental diseases as we used to. What we have learned from model systems about the molecular mechanisms of disorders of the developing nervous system can now be re-examined from this perspective. Autosomal recessive primary microcephaly (MCPH) is a rare genetic disease defined by a decrease in occipito-frontal head circumference at birth of greater than 3 standard deviations. Out of the Eight known loci linked to MCPH, five causative genes have been identified, encoding microcephalin, cyclin-dependent kinase 5 regulatory associated protein 2 (CDK5RAP2), abnormal spindle-like, microcephaly associated protein (ASPM), centromeric protein J (CENPJ), and SCL/TAL1-interrupting locus (STIL). Interestingly, all of these proteins localize to the centrosome during all or part of the cell cycle. In vitro studies in cultured cells have shown that these genes play essential roles in cell cycle progression and centrosome duplication. It has been hypothesized that these functions in turn regulate the ability of neural stem cell to produce neurons; however these hypotheses have not been tested in vivo. To investigate the role of microcephaly genes in neural stem cells during brain development, we used loss of function studies via gene knockdown by RNA interference (RNAi). Using intraventricular injection of constructs expressing CDK5RAP2 followed by *in utero* electroporation in mouse embryos at E12.5 or later, we observed the stem cell dynamic and their progeny subjected to RNAi with long-term timing-lapse imaging. We found that the classic interkinetic nuclear oscillation and cell division in radial glial cells were completely blocked, suggesting a novel mechanism for CDK5RAP2 in regulating neural stem cell self-renewal. Taken together, our observations indicate that MCPH genes CDK5RAP2 have pivotal functions in neocortical development. Understanding the cellular role of MCPH genes will shed light on pathogenesis of the microcephaly disease and related neural developmental disorders.

Poster Board Number: F-1089

SINGLE-CELL GENE EXPRESSION PROFILING IDENTIFIES PROGENITOR SUBCLASSES IN NEUROSPHERES

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Background Neural stem cells (NSCs) are cells of the central nervous system (CNS) which can self-renew and are multipotent. NSCs and neural progenitors (NPs) can be propagated as a free-floating 3D culture system known as neurospheres (nsphs). The NSC/NP composition of nsphs has been investigated retrospectively using morphological criteria, selection markers and proliferation assays. However, the exact cell-types present within nsphs are still unknown. Methodology and Principal Findings We used single-cell gene expression profiling (scPCR) of 48 genes in 187 cells each from passage 2 and passage 5 nsphs to identify cell-types. Using principle component analysis we identified three discrete cell populations in nsphs based on the expression of Notch1, Hes1, Hes5, FGFR2 and Pax6. The gene expression profile of these three populations correlate with a developmental timeline of early, intermediate and late NPs as seen *in vivo* from mouse brain. We found that the early NPs consist mostly of passage 2 cells while the intermediate and late NPs consist only of passage 5 cells. We enriched the cell population for neurosphere-forming cells (NFCs) using morphological criteria of forward scatter (FSC) and side scatter (SSC). FSC/SSChigh cells generated 2.35-fold more nsphs than FSC/SSClow cells at clonal density. FSC/SSChigh cells were enriched for NSCs and Lewis-X+ve cells, possessed higher phosphacan levels

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and were of a larger cell size. Clustering of both FSC/SSChigh cells and FSC/SSClow cells using non-metric multi-dimensional scaling (nMDS) identified a NFC cluster. Significantly, the gene expression profile of the NFC cluster could be linked to that of early NPs. Conclusions and Significance We have identified the gene expression profile for three cell populations that define nsphs. The three populations have gene expression profiles similar to that of early, intermediate and late NPs found *in vivo* respectively which suggests that nsphs can be used to model CNS development. We found that passage number affects the developmental state of npshs and that early NPs are the cell population that gives rise to nsphs. In future work it may be possible to further dissect the NFCs and reveal the molecular signature for NSCs.

Poster Board Number: F-1090

ASSESSMENT OF CERVICAL SPINAL CORD INJURY MODELS: COMPARISON AND VALIDATION USING EXTENSIVE NEUROBEHAVIOURAL ASSESSMENT AND STEM CELL THERAPY

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Thoracic rodent models of spinal cord injury (SCI) predominate as preclinical translational models; however, cervical SCI presents as the majority of clinical cases and represents the most significant personal impact and societal burden. The applicability and utility of thoracic and lower cervical SCI preclinical models was evaluated using clip contusion-compression injury paradigm in rats. Spinal injuries were realized in female Wistar rats at the C5, C6, C7 and T7 vertebral levels (controls: laminaectomy-only) using severe clip contusion-compression (28g for 60s). Animals were assessed for 10 weeks using: grip strength meter, Inclined Plane test, WARP contracture scale, BBB locomotor scale, bladder recovery, motor- and somatosensory-evoked potentials (MEP, SEP), and H reflex. LFB/H&E histological staining and T1-weighted MR imaging were used to assess tissue loss and cavitation. Adult neural precursor (aNPC) cells were transplanted (2x10^6 cells, or vehicle only) into C6-injured rats at 2 weeks post-injury and followed with similar neurobehavioural assessment. Spontaneous neurological recovery was observed with expected plateaus in all injury groups. Grip strength exhibited marked impairment with C5 and C6 injuries (291+/-38g and 566+/-80g, respectively, p 0.01), compared to C7, T7 and laminectomy-only shams (1315+/-65g, 1665+/-49g and 1130+/-50g, respectively) (p 0.0001). Inclined Plane test showed stratification between all groups in grasping and trunk support (p 0.05 between and p 0.0001 among groups). Contracture measured with WARP scale demonstrated significantly higher manual dexterity/utility in C6 compared to C5 rats (p 0.05). No differences were seen in BBB and Subscore. T1-weighted MR revealed similar and overt signal changes in all injuries, as confirmed with quantitative lesion analysis using LFB/H&E. Axonal conduction declined with ascending injury, as measured by peak latency and amplitude (p 0.01) with SEP and MEP. Forelimb spasticity was evident by H/M reflex ratios in C6 and C5 groups. Following aNPC transplantation in C6 animals, grip strength increased 2-fold compared to vehicle controls (417+/-59g vs. 211+/-55g, p=0.007), while Inclined Plane and BBB showed no difference (p=0.15 and 0.88, respectively). Electrophysiology revealed significant improvements to lower peak amplitude and latency in SEP of forelimbs in C6-injured rats compared to controls (p 0.05). The level of injury is highly determinant of forelimb deficit, and precludes using C7 or T7 level injuries to study cervical SCI. The C6 clip paradigm appropriately models neurobehavioural recovery and spasticity of cervical SCI, with utility as a preclinical cervical model exhibited by forelimb recovery following adult NPC transplantation.

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ACUTELY-ENGRAFTED NEURAL PRECUOSOR CELLS EXERTED NEUROPROTECTIVE EFFECTS ON SPINAL CORD INJURY BY THEIR NEUROHUMORSL SECRETIONS

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Neural precursor cells (NPCs)-transplantation is a promising treatment for various neurodegenerative disorders, including spinal cord injury (SCI). However, the mechanisms underlying functional improvement on SCI are not fully understood. Indeed, there are several possible explanations for the functional improvement through NPCs-transplantation: (1) synapse formation by graft-derived neurons; (2) remyelination by graft-derived oligodendrocytes; (3) growth support; (4) immunomodulation; (5) neuroprotection and so on. To determine which mechanism mainly contributes to functional improvement in acute transplantation, we transplanted NPCs harvested from embryonic mouse striatum into the injured spinal cord immediately after contusion injury, and evaluated the weekly recovery in the open-field score. In our study, the most prominent effect was observed within the first week after transplantation. During this time period, significant increases in the expression levels of several neurotrophic factors were observed, NGF, GDNF, BDNF, CNTF, IGF1, PDGF, VEGF in the NPCs-transplanted groups. In addition, these expression levels increased in proportion to the number of transplanted cells, indicating *in vivo* neurohumoral secretion by the engrafted NPCs. Histopathological examinations at 7 days after transplantation revealed fewer TUNEL-positive cells and more spared myelin in the NPCs-transplanted group compared to that in the medium-injected controls. Furthermore, we observed the activation of Akt and NF-kB, and a significant up-regulation of their downstream transcriptional targets, Bcl-2 and Bcl-xL, in the NPCs-transplanted group, resulting in decreased activation of caspases 9 and 3. While the increased expression of anti-inflammatory cytokines, such as IL-10 and TGFb was observed in the NPCs-transplanted group, no significant differences were observed in terms of the expression of pro-inflammatory cytokines or the number of infiltrating inflammatory cells. Consistently, Fas, FasL, and caspase 8 expression levels, the extrinsic apoptotic-cascade mediators induced by inflammation, did not significantly differ from that in the controls. These results indicate that engrafted NPCs ameliorate pathological changes during the acute phase of SCI, particularly the secondary damage cascades that are dependent on the mitochondrial apoptosis pathway, resulting in improved functional recovery.

Poster Board Number: F-1092

DIRECT REPROGRAMMING OF FIBROBLASTS INTO OLIGODENDROGLIAL CELLS

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Pelizaeus-Merzbacher disease (PMD) is an X chromosome-linked dymyelinating disease of the central nervous system (CNS) with a broad range of clinical severity that is caused by more than 60 known mutations in the proteolipid protein 1 (PLP1) gene. Like other neurodegenerative diseases, the study of pathogenesis of

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PMD is largely hindered by the lack of access to patient-derived disease-affected cells, oligodendrocytes in this case. One possible solution is the generation of iPS cells and subsequent glial differentiation. However, current technology to generate oligodendroglial cells from induced pluripotent stem (iPS) cells is difficult, variable and time consuming. Better differentiation strategies or alternative methods to generate oligodendroglial cells are in great need. Our group has recently shown that human fibroblasts can be directly reprogrammed into functional neurons by expression of the four transcriptional factors. This finding raised the possibility that modifications to our method would mediate the direct generation of induced oligodendroglial (iO) cells from skin fibroblasts. Here we show that forced expression of a combination of three transcription factors can efficiently convert mouse and rat fibroblasts into induced oligodendroglial cells within three weeks. More importantly, these iO cells robustly differentiate into myelinating oligodendrocytes when co-cultured with primary retina ganglion cells. The generation of iO cells may provide insights into the molecular nature of oligodendroglial differentiation and potential therapies for demyelinating diseases.

Poster Board Number: F-1093

THE BASAL PROCESS OF NEURAL STEM CELLS HELPS INTERKINETIC NUCLEAR MIGRATION AND NEURON/PROGENITOR TERRITORIALIZATION

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We are asking how neural stem cells' morphology is three-dimensionally regulated and how this regulation contributes to the overall brain formation. Stem/progenitor cells in the mammalian brain primordia originally take a neuroepithelial structure in which their nuclei/somata diffusely occupy the entire wall (about ten nuclei thick) of the neural tube or brain vesicle. This diffuse nuclear distribution is due to the cell cycle-dependent, to-and-fro nuclear movement (called interkinetic nuclear migration, INM) exhibited by each of the neuroepithelial cells (80 μm long) that span from the apical (inner/ventricular) surface to the basal (outer/pial) surface of the wall. When the first neuronal group comes out as a result of divisions within the initial neuroepithelium, neurons accumulate in an outer zone (1-2 cell thick) just beneath the basal lamina and stem/progenitor cells become longer (90-100 μm). The elongated stem/progenitor cells keep their apicobasal attachment as well as nuclear migration trajectory in a range of 80 μm (ten nuclei thick) with a basal/pial process (~20 μm) extended. How this elongation occurs is unknown and it is important to understand how this phenomenon might affect stem/progenitor cells' cytogenetic behavior. Through *in utero* electroporation-mediated RNAi experiments and live imaging in slice culture, we found that the earliest cohort of neurons in the developing mouse neocortex may play an important role in extrinsically shaping the neural stem/progenitor cells. Acute dysfunction of neocortical neurons that are born during E10-E11 resulted in the loss of Pax6-expressing undifferentiated progenitor cells' elongation and abnormal INM by E12 as well as disrupted territorialization between progenitors and neurons by E13, further showing massive histogenetic failures by E15.

Poster Board Number: F-1094

SIGNIFICANCE OF REMYELINATION BY TRANSPLANTED NEURAL STEM/PROGENITOR CELLS TRANSPLANTED INTO THE INJURED SPINAL CORD

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(Background) Previous reports including ours demonstrated that stem cell based therapy could be used to treat patients with spinal cord injury (SCI). More research is needed, however, to understand the mechanism of functional recovery after cell transplantation. Here we sough to determine the extent how graft-derived-remyelination contributes to functional recovery. To isolate the effect of remyelination from other possible mechanism of functional recovery, we took advantage of NS/PCs obtained from myelin-deficient shiverer mutant (shi) mice, which behave like wild-type NS/PCs in vitro and *in vivo*, except for their myelinating potential. (Methods) NS/PCs were obtained from striatum of E14.5 shi mice embryos (shi-NS/PCs) and wild type mice (wt-NS/PCs). In vitro proliferation and differentiation assays, and a PCR assay for trophic factors were performed. shi-NS/PCs or wt-NS/PCs were grafted into the injured spinal cord of adult NOD/SCID mice 9 days after injury and motor function was evaluated for 7 weeks. Survival rate of transplanted NS/PCs was also evaluated by bioluminescence imaging (BLI) followed by electrophysiological and immunohistological analyses. (Results)shi-NS/PCs differentiated into Tuj1+ neurons, GFAP+ astrocytes, and CNPase+ oligodendrocytes, but not into MBP+ oligodendrocytes in vitro. Both grafted NS/PCs showed similar survival, which was confirmed by BLI analysis. Consistent with in vitro differentiation assay, shi-NS/PCs differentiated into Hu+ neurons, GFAP+ astrocytes, and APC+ oligodendrocytes but not into MBP+ oligodendrocytes *in vivo*. Immuno-EM analysis revealed that shi-NS/PC-derived oligodendrocytes could form myelin sheaths, which were much thinner than those of wt-NS/PC-derived ones. The shi-NS/PCs grafted animals showed some locomotor and electrophysiological recovery, which was significantly less than those in the wt-NS/PCs grafted animals. (Conclusion) These findings suggested biological significance of remyelination by graft-derived oligodendrocytes in terms of functional recovery after NS/PCs transplantation for SCI.

Poster Board Number: F-1095

DOWNREGULATION OF NOTCH MEDIATES THE SEAMLESS TRANSITION OF INDIVIDUAL *DROSOPHILA* NEUROEPITHELIAL PROGENITORS INTO OPTIC MEDULLAR NEUROBLASTS DURING PROLONGED G1

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Neurogenesis has been viewed as a process in which epithelial cells produce nonepithelial cells. During development of the *Drosophila* optic medullar primordium, symmetrically dividing neuroepithelial cells (NEs) first expanding themselves and asymmetrically dividing neuroblasts (NBs) arise subsequently. However, It is still unclear that what mechanisms specify the NEs to change into NBs. Here we provide evidence that Notch signaling is necessary for the inhibi-

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tion of NEs in their undifferentiated state, thereby preventing their precocious transition to NBs. In this study, we performed detailed analyses demonstrating that individual NEs converted into NBs during the multistep transition. The 2 or 3 rows of cells between the fully undifferentiated NEs and mature NBs were revealed to be the characteristic drop-shaped cells. They had weak signal of the neural progenitor gene product Asense (Ase) that determined the differentiated status as if they had just started to express it. From the series of BrdU incorporation experiments, we could show that this transition occurred during an elongated G1 phase. During this G1 phase, each columnar NE changed morphological features dynamically. In addition, both the expression levels and localization pattern of ligand Delta as well as receptor Notch were dramatically altered during the transition period. Once the NE-to-NB transition was complete, the former NE changes its cell-cycling behavior, commencing asymmetric division. Moreover, we found that Notch signaling was activated just before the transition, which accompanies the Su(H)-dependent downstream gene activation and was rapidly downregulated. The involvement of Notch in the NEs was further investigated by the genetic disruption analysis. When the homozygous Notch clones were occurred in the NE region of optic primordia of larval brain, the precocious onset of transition occurred. As for Delta, the ectopic expression and localization changes including cytoplasmic accumulation were observed in the actual sequence of maturity, instead the timing was too early than the surrounding region. Taken together, activation of Notch signaling during a finite window coordinates the proper timing of the transition of NEs to NBs. We also obtained new evidence that this transition is mediated by the downregulation of Notch through a feedback loop with Delta. Our results suggest that the morphological transition involves a delay in cell-cycle progression and Delta-Notch signaling. Although this may seem to be a surprising role for Notch, the Notch signaling system is required repeatedly during development, and the large number of mediators and modulators associated with this pathway lead to a great variety of biological outcomes.

Poster Board Number: F-1096

ANTENATAL EXPOSURE TO SUBEROYLANILIDE HYDROXAMIC ACID PROMOTES NEUROGENESIS AND ALTERS NEURAL PROGENITOR CELL FATE SPECIFICATION IN THE DEVELOPING BRAIN

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In mouse, the production of projection neurons derived from neural progenitor cells (NPCs) occurred between embryonic day 11 (E11) and E17, in which the first wave neurons generated are Cajal-Retzius cells which will settled in the most superficial cortical layer (layer I). Neurogenesis proceeds subsequently in an inside-out fashion: mid-gestational NPCs (E11-E14) give rive to neurons located in deep-layers (layer V-VI) characterized by the expression of Tbr1, Ctip2 and its upstream regulator Fezf2, and then the residual NPCs (E15-E17) generate neurons in superficial-layers (layer II-IV) which express Satb2 or Cux1. We have previously reported that the specification and differentiation of NPCs are regulated by, at least in part, an epigenetic mechanism, histone acetylation. An increase and decrease of histone acetylation are mediated by histone acetyltransferases and histone deacetylases (HDACs), respectively, contributing crucially to regulations of gene expression. The presence of HDAC inhibitors (HDACi) such as valproic acid (VPA) and trichostatin A in NPCs culture has been shown to attain a tendency for neuronal over glial differentiation. VPA treatment to mouse embryonic stem cells-derived NPCs increased the production of Cux1+ superficial-layer neurons and decreased Ctip2+ deep-layer neurons. However, the effect of HDAC inhibition *in vivo* on the fate specification of NPCs within the developing mammalian cortex is largely unknown. Another HDACi, suberoylanilide hydroxamic acid (SAHA) is currently considered to be one of the promising anti-cancer drugs. However, in preclinical study using animal models, it has been reported that SAHA crosses the placenta and can be found in fetal plasma at levels up to 50% of maternal concentrations, implying that the behaviors of cells in fetuses are influenced by this drug through the medication of the mothers during their pregnancy. Nevertheless, there has been no report of SAHA in this aspect, including its effects on NPC regulation during brain development. To assess whether SAHA influences the regulation of NPCs in the embryonic brain, SAHA or methyl-cellulose (control) was orally administered to pregnant mice, once a day for three consecutive days starting from E12 until E14, a period when cortical neurogenesis is prominent and fate switch of NPCs from deep- to superficial-layer neurons occurs. After prenatal HDAC inhibition by SAHA, acetylation of histone H3 and H4 was up-regulated compared to control mice. SAHA-treated mice also showed an enlargement of cortical plate thickness and enhancement of cortical neurogenesis as judged by the expression of neuronal markers, β-III-tubulin and doublecortin. Moreover, we also found that SAHA down-regulates expression of genes specific for deep-layer neuronal lineage (Fezf2, Ctip2), while it up-regulates genes for superficial-layer lineage (Satb2, Cux1). Taken together, these results suggest that SAHA switches the fate of NPCs from deep- to superficial-layer neurons during development of the cerebral cortex.

Poster Board Number: F-1098

NEWLY IDENTIFIED MINP, MIGRATORY INHIBITORY PROTEIN REGULATES RADIAL MIGRATION RATE IN THE DEVELOPING NEOCORTEX

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The mammalian cerebral cortex has six-layered structure that contains diversity of neurons. Among the mechanisms governing the cerebral cortex construction, neuronal migration is the most crucial and a fundamental process in the development of nervous system. Impairment in migration results in structural defects that are accompanied by mental abnormalities such as Disc1 mutation in schizophrenia. Radial migration of newborn neurons is regulated by multiple intracellular and extracellular signals that are involved in some molecular pathways and cellular events. Although more positive cues of radial migration have been already unveiled, the negative regulatory mechanisms remain obscure. Given that neuronal migration is the consequence of the sum of positive and negative regulatory mechanisms, migrating neurons must also respond to lesser known negative cues that limit migration machinery. Here we report newly identified protein, MINP (Migration Inhibitory Protein) that regulates radial migration rate. MINP gene is located in chromosome 16p13 whose copy number variations are highly associated with several neurodevelopmental disorders such as mental retardation, schizophrenia, AD/HD and epilepsy. We first confirmed mRNA expression pattern of MINP during developmental ages of mouse from embryonic day 12.5 till adult. MINP is highly and specifically proved to be expressed in matured neurons of cortex, hippocampus and thalamus in the embryonic mouse brain and spinal cord in CNS as well as PNS by in situ hybridization

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and RT-PCR. The peak of expression is observed around birth and decreased in adult nervous system. Then we established polyclonal antibody against this new protein and reconfirmed its expression in matured neurons that are double-positive with NeuN and βtubulin-III by immunohistochemistry. After verification of siRNA knockdown and overexpression vector's protein production, we applied them to *in utero* electroporation technique for in vivo experiment and cortical precursor culture system to in vitro experiment. Knockdown and overexpressed MINP showed no change of proliferation, differentiation and cell survival of cortical precursors either in vitro or in vivo. However, knockdown of MINP shows that the neuronal migration is accelerated while overexpression is prone to inhibit it. According to the final destination of neuronal migration does not seem disordered by longer observation, we concluded this protein specifically regulates neuronal migration speed negatively. Although molecular mechanism of MINP has not been clarified yet, mutant mouse of this gene may show interesting phenotype that mimics phenotypes of patients with neurodevelopmental diseases.

Poster Board Number: F-1099

MIR-195 INDUCES APOPTOSIS IN HUMAN EMBRYONIC STEM CELL DERIVED NEURAL PROGENITOR CELLS THROUGH A NOVEL TARGET

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Neural progenitors (NPCs) derived from human embryonic stem cells (ESCs) could be used to treat neural degenerative diseases. It is important to understand how proliferation and apoptosis of NPCs are regulated at a molecular level. miR-195 is known highly expressed in the brain and it is reported involved in a variety of pro-apoptosis or anti-apoptosis processes in cancer cells. However, the role of miR-195 in early neural development remains elusive. In this study, we investigated the role of miR-195 for the control of apoptosis in NPCs, which were derived from human ESC lines (SHhES1 and H9) by different methods. We found that over-expression of miR-195 precursors in NPCs could induce extensive apoptotic cell death, resulting in a marked increase in the activated Caspase 3 protein level and in the number of cells positive in Annexin V and TUNEL analyses. Conversely, its antagonist miR-195 LNA rescued the phenotype. Consistently, our transcriptional microarray data obtain from miR-195 over-expressed NPCs indicated that the major function of miR-195 is for the regulation of cell apoptosis rather than differentiation. Mechanistically, our results suggested a small GTP binding protein ARL2 might be the direct target of miR-195 and responsible for the apoptosis induced by miR-195 in human NPCs. In addition, we found that the other member of miR-195 cluster, miR-15a, also promoted apoptosis in NPCs. Thus, our study reveals a previously unappreciated role of miR-195 for the regulation of apoptotic cell death and establishes the molecular and functional link between miR-195 and ARL2 in NPCs.

Poster Board Number: F-1100

THE ROLE OF MICRORNA LET 7D IN NEUROGENESIS

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MicroRNAs have been shown to play important roles in neural development. However, the role of microRNA let-7d in neurogenesis remains unknown. We show here that let-7d is expressed in mammalian brains and exhibits increased expression upon neural differentiation. Let-7d regulates neural stem cell proliferation and differentiation by targeting the stem cell regulator TLX. *In utero* electroporation of let-7d to neural stem cells in embryonic brains led to reduced cell proliferation, premature differentiation and outward migration of the transfected cells. In addition, let-7d inhibits the expression of Lingo-1 and enhances neuronal maturation. Intracranial transduction of let-7d led to enhanced dendritic growth of neurons in adult mouse hippocampus. Let-7d, by targeting TLX and Lingo-1, establishes a novel strategy to control multiple steps of neurogenesis, from neural stem cell proliferation and differentiation to neuronal maturation.

Epithelial Cells (Not Skin)

Poster Board Number: F-1101

MICROARRAY ANALYSIS OF ORAL EPITHELIAL CELL SHEET FOR CORNEAL REGENERATION

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Corneal defects caused by unilateral/bilateral limbal stem cell deficiency (LSCD) resulting from trauma, such as chemical burn and eye diseases, have been successfully treated with transplantable cultured limbal stem cell sheets cultured on temperature-responsive cell culture ware. In bilateral LSCD, oral mucosal epithelial cells were used as a cell source to engineer the transplantable cell sheet. The goal of the present study is to determine the effects of cell culture conditions and to characterize the phenotype of the cells that form the engineered cell sheet for cornea regeneration. Rabbit oral mucosal epithelial cells were isolated and cultured, and a transplantable cell sheet was engineered. RNAs were isolated from the cells used as a starting point and from the engineered cell sheet (n=4). Microarray analysis and comparisons between the two groups of cells were performed. Out of 43K rabbit probes listed on the microarray chip, only 160 genes were identified and were known, which represents a valuable results since the rabbit genome is not fully sequenced. Genes from ECM-receptor interaction pathway, which is responsible for cell adhesion, migration, differentiation, and proliferation, were identified. Matrix metalloproteinase (MMP1, MMP3, MMP12 and MMP13) and collagen proteins were identified and were changed, reflecting the engrafting potential of the engineered cell sheet. We also identified genes from the adherens junction pathway, which are responsible for maintaining tissue architecture and cell polarity, and limit cell movement. Gap junction membrane channel protein alpha 7 (GJA7, known as Connexin-45) was found up regulated, indicating the established cell-cell interaction in the engineered cell sheet. Inflammatory marker interleukin 1 beta (IL-1 beta) was found down regulated and the enzymes involved in protecting the cornea against oxidative damage, such as alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH), were found up regulated. In conclusion, microarray analysis approach allowed us to identify several rabbit cornea genes in the cultured oral mucosal epithelial cell sheet, and allowed us to investigate the expression of genes potentially beneficial for successful cornea regeneration.

Poster Board Number: F-1102

THE STEM CELL LINEAGES AT THE ORIGIN OF HAIR FOLLICLE RENEWAL

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Mammalian hair represents a paradigmatic model system for studying adult stem cells, as it undergoes cyclical degeneration,

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quiescence and renewal. Hair follicles are believed to renew themselves from a pool of long-lived stem cells giving rise to all epithelial layers of the hair during each growth cycle. Currently, this hypothesis is common to all models of hair follicle renewal. Recent genetic lineage tracing provide a detailed molecular characterisation of the region believed to contain the long-lived stem cells - the bulge - and uncovered an evident assortment of diverse keratinocytes with stem cell properties located in distinct bulge areas and expressing different markers, such as CD34, Lgr5, Sox9 and others. By taking advantage of the hair follicle cycling properties, we designed strict lineage-tracing experiments to determine the fate and source of hair follicle stem cells involved in renewal throughout normal homeostasis. We used clonal analysis to determine the individual contributions of the hair follicle stem cells, regardless their genetic profile. Our results reveal an heterogeneity of the hair follicle stem cells in their individual contribution to the renewed hair follicle. These findings imply the existence of precursors contributing only to some or even a single layer of the hair follicle. We further determined the cellular organisation of the hair follicle outer layer by *in vivo* clonal analysis by 3D imaging of the clones. Thus, the clonal hierarchies led us to establish a novel model of the hair follicle growth and organisation and to re-evaluate the nature and role of the hair follicle sub-populations responsible for renewal.

Poster Board Number: F-1103

SELECTIVE ACTIVATION OF ESTROGEN RECEPTOR B IMPAIRS PROSTATE REGENERATION

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Regenerative stem/progenitor cells are potential therapeutic targets for prostate diseases; however, they evade current treatments. Androgen depletion, the most common treatment for benign prostatic hyperplasia and prostate cancer, only eliminates the differentiated epithelium. The remaining castrate-resistant basal cells, which contain a subpopulation of stem/progenitor cells, can fully regenerate the prostate gland if androgens are restored. We have recently shown that estrogens also regulate the survival of prostate cells using a selective agonist (8β-VE2) that activates estrogen receptor β (ERβ), but not estrogen receptor α (ERα). Unlike castration, treatment with the agonist induces apoptosis of basal cells in mouse prostate tissue. Importantly, the mechanism of action of 8β-VE2 is different from castration, inducing TNFα-dependent apoptosis through the intrinsic signalling pathway. Therefore, we investigated whether selective activation of ERβ inhibits prostate regeneration by targeting stem/progenitor cells. We compared the effects of short-term castration or ERβ agonist treatment followed by a recovery period where the prostate gland was allowed to regenerate. The prostate tissue of castrate-recovery mice returned to normal, whereas regeneration was impaired in ERβ agonist-treated mice. Agonist-treated tissues displayed abundant cystic atrophy and accumulation of mucopolysaccharide secretions within glands. We next used an *in vitro* prostasphere assay to determine whether 8β-VE2 blocks regeneration by targeting stem/progenitor cells. Compared to the vehicle control, 8β-VE2 reduced the size and number of spheroids that formed from mouse prostate tissue. Moreover, subsequent self renewal was also inhibited, as observed through a decrease in the formation of secondary spheroids in the absence of 8β-VE2 treatment. These data demonstrate that ERβ activation inhibits prostate regeneration, providing a rationale for the combined therapeutic use of androgen-deprivation and selective ER agonists for the treatment of prostate diseases.

Poster Board Number: F-1104

MICRORNA-184 TRIGGERS EPITHELIAL STEM CELL DIFFERENTIATION BY REPRESSING THE STEM CELL MARKER CYTOKERATIN 15

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microRNAs (miRs) play a role in various physiological processes including embryogenesis, tissue regeneration and diseases. Many attempts have been made to identify factors that are specifically expressed by epidermal and limbal stem/progenitor cells related to tissue homeostasis. Here, we report that miR-184 plays a general role in regulating epithelial stem cells differentiation. *In situ* hybridization analysis revealed that miR-184 is not present in putative corneal and epidermal stem cell compartments that are hallmarked by cytokeratin 15 (K15), while its expression increased during epithelial differentiation in vivo and in vitro. We found that miR-184 directly represses K15 production and accelerates early cell differentiation. Moreover, we observed an increase of miR-184 expression coupled with a decrease in K15 production in pannus (abnormal corneal vascularization) of patients with limbal stem cell deficiency. Accordingly, a point mutation in the seed sequence of miR-184 has been recently found in corneal and lens diseases. Given that over expression of miR-184 in limbal stem cell-enriched culture resulted in a dramatic decrease in clonogenic potential, our data indicate that miR-184 expression induces an escape from stemness state and suggest that abnormal expression of miR-184 may lead to a decline in limbal stem cell pool. In addition, miR-184 expression displayed a major inhibitory effect on both the expression of the corneal stratification marker, K12 and the stratification ability of corneal epithelial cells in organotypic culture. Given that miR-184 is not expressed in the upper layers of the corneal epithelium which are enriched with K12, we propose that the decrease in miR-184 allows terminal differentiation. Taken together, we propose that miR-184 is the guardian of epithelial progenitor cells; on one hand, its elevation promotes stem cell commitment into transit amplifying progenitors, while its further decline modulates terminal differentiation.

Poster Board Number: F-1105

TRANSCRIPTION FACTOR OCT1 IS A SOMATIC AND CANCER STEM CELL DETERMINANT

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Defining master transcription factors governing somatic and cancer stem cell identity is an important goal in understanding the role of stem cells in normal and disease states. Here I show that Oct1, a transcription factor implicated in stress responses, metabolic control and poised transcription states, regulates normal and pathologic stem cell function. Oct1HI cells in the colon and small intestine express stem cell markers. In primary malignant tissue, high Oct1 protein but not mRNA levels correlate with the frequency of CD24LO/CD44HI cancer initiating cells. Modulating Oct1 alters the proportion of ALDH HI and dye efflux HI cells, and cells isolated on the basis of high ALDH activity harbor elevated Oct1 protein but not mRNA levels. In addition to previously described targets, I have identified four Oct1 targets associated with the stem cell phenotype. Functionally, I have shown that Oct1 promotes tumor engraftment frequency and hematopoietic stem cell engraftment potential. The data indicate that Oct1 regulates normal and cancer stem cell function.

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Poster Board Number: F-1106

DIFFERENTIATION AND GENERATION OF FUNCTIONAL UROTHELIAL CELLS FROM HUMAN EMBRYONIC STEM CELLS

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Background: Repair and regeneration of damaged or diseased bladder is vital for patients with congenital bladder disease or cancer. Generation of urothelial cells from hESC or iPS cells would facilitate further research of urothelial differentiation as well serve as a source for cell and tissue replacement for patients with urological disease. The present investigation is the first of its kind to successfully differentiate hES cells into urothelial cells. Methodology / Principal Findings: In this study, we developed and describe generation of urothelial cells from hESC by modulating the culture conditions and analyzing the transcription regulators that are involved during the differentiation of urothelium. During embryonic development of bladder, the inner layers of bladder cells originate from definitive endoderm through hind gut cloacae invagination. We developed the method using specific culture conditions, such as media, serum and growth factors that efficiently differentiate hES cells into definitive endoderm. DE further induced through the cytodifferentiation process into urothelial cells by induction of PPARy. The generated urothelial cells were validated by urothelial specific immune markers and RT-PCR techniques. Under the stimulatory condition, the molecular markers of DE, such as Sox17 and FoxA2, exponentially increased, whereas, during differentiation of DE into urothelium, the specific markers of urothelium (uroplakin subtypes UP1a, 1b, 2 and 3) significantly increased and DE markers gradually decreased. Regenerated urothelial cells derived from GFP transduced hES cells were orthotopically transplanted into NOD/SCID mice by microinjection of cells under the epithelium of recipient bladders. At different time intervals after transplantation, human GFP-labeled cells were identified and cellular incorporation into the bladder was verified by fluorescent immuno markers. Conclusion: The described *in vitro* method to induce differentiation of hES cells into urothelium demonstrates the ability to produce urothelium from hES cells and the feasibility of producing autologous urothelium from iPS cells in the future. The presented orthotopic cell transplant model demonstrates that these cells do functionally integrate and could potentially be used for treatment of bladder disorders.

Poster Board Number: F-1107

MAJORITY OF PROXIMAL TUBULAR CELLS IN THE OUTER MEDULLA ARE SLOW-CYCLING AND EQUALLY CONTRIBUTE TO TUBULAR REGENERATION AFTER RENAL ISCHEMIA

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INTRODUCTION AND AIMS: Renal proximal tubular epithelium has a capacity to regenerate after a variety of insults. During tubular recovery after injury, survived tubular cells acquire immature phenotype, proliferate, migrate and finally differentiate into matured tubular epithelium. Using an *in vivo* bromodeoxyuridine (BrdU) labeling, we previously identified label-retaining cells (LRCs), which act as the source of proliferating cells after injury, in renal tubules of normal rat kidney (J Am Soc Nephrol 14: 3138-3146, 2003) and found that LRCs possess renal progenitor-like property (J Am Soc Nephrol 17: 188-198, 2006). However, it remains unknown whether label-retaining potential is limited to a specific cell population or not. To clarify this issue, we examined the presence of LRCs in normal rat kidney using two kinds of thymidine analogues, iododeoxyuridine (IdU), and chlorodeoxyuridine (CldU). METHODS: 1) Long labeling experiment: Using osmotic pomp, BrdU was continuously given into 7-week-old Wistar rats for one, two, three, and four weeks and the number of BrdU-positive cells was analyzed. 2) Double labeling experiment: IdU and CldU were sequentially administered for 7 days into rats (100 mg/kg/day each) with 3 days interval. At two months after CldU labeling, ischemia/reperfusion injury was induced in these rats and kidneys were removed for histological analysis at 24h after reperfusion. Using anti-IdU Ab (that recognizes IdU, but not CldU) and anti-CldU Ab (that recognizes CldU, but not IdU), two LRC populations (LRC-IdU and LRC-CldU) were identified and the numbers of them were analyzed. RESULTS: Long labeling experiment demonstrated that the number of BrdU-positive tubular cells was positively associated with labeling period. Majority of proximal tubular cells in the outer medulla of the kidney became BrdU-positive after 4-week labeling. Double labeling experiment showed that LRC-IdU and LRC-CldU were scattered in renal tubules, but were not co-localized. The numbers of each LRC was similar and significantly increased after injury. There was no significant difference in the ratio of cell division among these LRCs after ischemia. CONCLUSIONS: These findings suggest that the majority of proximal tubular cells in the outer medulla are slow-cycling and equally contribute to tubular regeneration after renal ischemia.

Poster Board Number: F-1108

DYNAMIC EXPRESSION OF CXCR4 AND CXCL12 IN THE DENTAL STEM CELL NICHE OF MOUSE INCISOR

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Rodent incisors are a type of continuously growing teeth that undergoes cyclical regeneration throughout their life. Dental stem cells located at the proximal ends of incisors, produced enamel formation cells with their stem cell niche. This niche consists of bulbar like epithelial tissues: apical bud and mesenchyme surrounding the epithelium. Multiple molecular signaling pathways control the maintenance of the dental stem cell niche. Growth factors, receptors and extracelluar matrices (ECMs) are thought to be the components of the dental stem cell niche which control spatiotemporal gene expression levels via epithelial - mesenchymal interactions. The chemokine receptor, CXCR4, is a seven- transmembrane spanning G- protein-coupled receptor for the CXCL12 ligand. Signals mediated by CXCR4/CXCL12 regulate cell migration, cancer metastasis, hematopoiesis and organogenesis. However, the functions of CXCR4 and CXCL12 in tooth development have not been elucidated. In this study, we examined the differential expression levels of CXCR4 and CXCL12 mRNA in the developing mice incisors and further investigated their functions in the epithelial dental stem cell niches. First, we conducted histological analysis by *in*

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situ hybridization. CXCR4 mRNA was found to be expressed in the inner enamel epithelium (IEE) at E14, then gradually restricted to the labial side of IEE in the apical bud by E18. While, the transcripts of CXCL12 existed in the IEE of the apical bud, pre-ameloblasts and mesenchyme beside the apical bud at PN4. CXCL12 mRNA expression levels increased from E14 to PN4 as detected by real-time PCR. CXCR4 deficient mice have short incisors and form epithelial cell aggregates in the apical bud. Furthermore, cellular proliferation marker Ki-67 positive cells were significantly reduced in the apical bud in comparison to wild type. Next, we used an epithelial dental cell line (mHAT9a) and small hairpin RNA specific for CXCR4, for the knock down (KD) experiments used for in the vitro assay. We evaluated the cellular proliferation of control and CXCR4KD mHAT9a cells cultured with CXCL12 recombinant protein in vitro. No significant differences in proliferation were observed in the presence or absence of CXCL12. Howevwe, CXCR4 KDs were found to suppress cellular proliferation. The transwell cell migration assay revealed control mHAT9a cells migrated toward CXCL12 proteins, while CXCR4 KDs exhibited impaired of cell motility. Therefore, we compared the differential expression of genes related to cell adhesion molecules and ECMs between CXCR4 KDs and controls. Some candidate genes were found to be dramatically increased in the CXCR4 KDs. These findings suggest that CXCR4 plays a significant role on the cell dynamics within the dental stem cell niche.

Poster Board Number: F-1109

PRINCIPLE CELL POPULATIONS IN THE BOVINE MAMMARY GLAND: DELINEATION OF CELL HIERARCHY AND LINEAGE COMMITMENT

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The role of somatic stem cells and their progenitors in mammary gland development and renewal has been extensively studied in the human breast and in the mouse model. Novel insights on the hierarchy of mouse mammary epithelial cells have recently been raised. In contrast, limited information is available on stem cells and their progeny in the mammary glands of other species. The aim of this study was to characterize the cell hierarchy and properties of distinct epithelial cell populations in the bovine mammary gland. This highly productive organ maintains extensive cell expansion and differentiation as well as comparable basic characteristics with the human breast. Collectively, these properties imply high compatability for studying mammary cell hierarchy and its diversity among mammalian species. Lin^- epithelial cells were sorted according to expression of CD24 and CD49f into four populations: $CD24^{med}CD49f^{pos}$ (putative stem cells, puStm), $CD24^{neg}CD49f^{pos}$ (Basal), $CD24^{high}CD49f^{neg}$ (putative progenitors, puPgt) and $CD24^{med}CD49f^{neg}$ (luminal, Lum). These populations maintained differential gene expression of lineage markers and markers of stem cells and luminal progenitors. High expression of Stat5a was observed in the puPgt cells. Notch1, Delta1, Jagged1 and Hey1 were mainly expressed in the puStm and Basal populations. Cultured puStm and Basal cells formed lineage-restricted basal or luminal clones and after re-sorting, colonies that preserved a duct-like alignment of epithelial layers. In contrast, puPgt and Lum cells generated only luminal clones and unorganized colonies. Under non-adherent culture conditions, the puPgt and puStm populations generated significantly more floating colonies. The increase in cell number during culture provides a measure of propagation potential, which was highest for the puStm cells. In conclusion, this study demonstrates for the first time that bovine cell populations acquire a conserved cell hierarchy paradigm similar to that delineated for their mouse and human counterparts: stem cells give rise to bi-potent progenitors that differentiate along the basal/ myoepithelial lineage and possibly also give rise to luminal cells. The stem cells also generate luminal-restricted progenitors that give rise to terminally differentiated cells. Importantly, some of the bovine cell properties that were involved in the definition of this hierarchy are novel or non-overlapping with mice or humans due to distinct characteristics of the bovine mammary cells. Finally, combined detection of ALDH activity with surface-marker analysis identified a small candidate stem cell-enriched subpopulation, representing 0.35% of the bovine mammary epithelial cells. Identifying the properties of bovine stem cells and their progenitors will undoubtedly promote our understanding of the bovine mammary gland's adaptation to high and continuous milk production and its possible resistance to tumorigenesis.

Liver Cells

Poster Board Number: F-1111

DIRECTED DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS INTO FUNCTIONAL HEPATOCYTES USING THE COMBINATORIAL CELL CULTURE PLATFORM COMBICULT™

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Pluripotent stem cells have the potential to offer a consistent, limitless supply of human hepatocytes for critical *in vitro* drug toxicity studies. However, generating functional hepatocytes from human embryonic stem (hES) cells remains a major challenge due to inefficient differentiation protocols, characterized by low yields, cellular heterogeneity and lack of functional attributes typical of primary hepatocytes. We previously utilized our CombiCult technology, a multiplexed screening platform, to screen 3,375 protocols in parallel to discover novel serum-free protocols for the differentiation of hES cells into hepatocytes. This resulted in the identification of 9 validated, serum-free protocols which generate hepatic-like cells exhibiting the morphology, marker expression, and some functionality typical of hepatocytes. We then performed a secondary screen of a further 2,700 combinations of growth factors and small molecules in order to identify additional cell maturation conditions. Induction of key CYP enzymes was used as readout of the screen in order to identify protocols that improve critical functionality of the hepatic-like cells derived from the first set of protocols. Cells from several of our original protocols were multiplexed into this maturation screen. This sequential use of Combicult screens enabled us to discover a number of high-value protocols for the induction, differentiation and maturation of hES cells into hepatic-like cells in serum-free conditions in a step-wise manner. Cells generated using these protocols are characterized by high yields, homogeneous populations and the morphology, marker expression and function of authentic hepatic cells and therefore promise to be a valuable tool for regenerative medicine and drug discovery applications.

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Poster Board Number: F-1112

MASS DIFFERENTIATION OF HUMAN INDUCED PLURIPOTENT STEM CELL INTO HEPATOCYTE LIKE CELLS IN STIRRER BIOREACTOR AND THEIR FUNCTIONAL CHARACTERIZATION IN CCL4 INDUCED LIVER INJURY IN MOUSE MODEL

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Due to lack of adequate donors for whole liver transplantation, cell therapy is a promising alternative for patients suffering from end stage liver disorders. Mass production of functional hepatic cells is the main challenge in cell based therapeutics. Automated culture of candidate cells and their direct differentiation can provide favorite cells in clinical scale. Optimization of dynamic status to increase the yield as well as replacement of expensive growth factors with small molecules can improve the harvest and make it more practical and feasible. Suspension culture of stem cells in spheroid form and direct differentiation of them into definitive endoderm (DE) was a crucial step in our protocol. We applied lower concentration of Activin A in correlation with Rapamycin to obtain the desired DE cells in a stirrer flask. This protocol can be considered as a new method for DE production. Then, DE spheres were moved to hepatocyte culture medium which enriched with Hepatocyte Growth Factor (HGF) and Fibroblast Growth Factor-4 (FGF-4) to produce hepatoblast cells. After 8 days, Dexamethasone and Oncostatin M were added into the induction medium to precede the maturation process of hepatoblasts cells into Hepatocyte-like Cells (HLCs). Quantitative RT-PCR was done to evaluate the expression of hepatic specific genes e.g. Alb, Afp and etc. Immunofluorescence study showed ALB and AFP expression in our cells. Functional assays for ICG uptake and release as well as PROD test have been performed successfully. PAS staining demonstrated efficient glycogen storage. Urea synthesis and detoxification abilities were presented. The HLCs were enriched via MACS according to the presence of ASGR-1 on their surface. Obtained cells were transplanted transdermally into CCL4 injured mice through spleen after their immunosuppression. Histological sections revealed sufficient homing of transplanted cells. Survival rate in cell transplanted mice was considerably higher than corresponding control and sham groups. Plasma levels of ALT and AST in cell-treated mice were lower; however the concentration of total albumin was higher in comparison with other groups. We hopefully consider this approach in production of HLCs as a new horizon in producing functional cells in clinical scale.

Poster Board Number: F-1113

USING HUMAN IPSCS TO MODEL LIVER DISEASE IN ALPHA-1 ANTITRYPSIN DEFICIENCY

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Alpha 1-antitrypsin (AAT) deficiency is the most common genetic cause of liver disease in neonates and children. It is an inherited metabolic disorder in which mutations in the coding sequence of the serine protease inhibitor AAT cause misfolding of the protein. These misfolded glycoproteins form aggregates, which accumulate in hepatocytes. This is believed to lead to programmed cell death, hepatic inflammation, fibrosis, and cirrhosis. The exact mechanistic role of the polymerized conformation of mutant AAT in the pathophysiology of liver disease is controversial and still being investigated. Attention has recently emerged on how these hepatocytes may respond to misfolded and aggregation-prone AAT, with a focus on cellular disposal pathways and stress response, including the autophagy pathway. In addition, the clinical presentation of liver disease in AAT deficient patients is variable. Only a fraction of affected individuals develop clinically significant liver disease, and therefore genetic and environmental factors must have a profound influence on the hepatic phenotype of this deficiency. We propose to generate a human induced pluripotent stem cell (iPSC) model of AAT deficiency by reprogramming fibroblasts of patients with various degrees of liver disease. We will analyze hepatocytes derived from AAT deficient iPSC to explore; 1) the relationship between AAT deficiency phenotype and early events leading to hepatocyte dysfunction, and 2) the existence of genetic modifiers that may alter the course of hepatocyte dysfunction as a result of the same AAT deficiency phenotype. Our study has the potential to aid in the design of new therapies for AAT deficiency, and also increase our understanding of the factors that make some patients more prone to develop hepatic dysfunction as a result of AAT deficiency and other liver diseases.

Poster Board Number: F-1114

THE APPLICATION OF HUMAN IPSCS AS AN *IN VITRO* EXPANSION SYSTEM IN GENERATING PURIFIED HEPATIC STEM/PROGENITOR-LIKE CELLS TO DETERMINE THE MOLECULAR MECHANISMS REGULATING HUMAN LIVER ORGANOGENESIS.

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Introduction Hepatoblasts, hepatic stem/progenitor cells during liver development, have high proliferative potential and bi-potent differentiation capacity into hepatocytes (hepatic parenchymal cells) and cholangiocytes. Proliferation and differentiation of hepatoblasts are regulated by soluble factors and interactions with non-parenchymal cells in fetal livers, i.e. fibroblasts, mesothelial cells, and endothelial cells. However, the molecular mechanisms regulating human hepatoblasts remain unknown because the *in vivo* analyses of human cells are difficult. To this end, induced pluripotent stem cells (iPSCs) generated from human cells are potentially good sources of understanding human liver development. In this study, we aim to establish an in vitro assay system of human liver development by purifying cultured hepatic stem/progenitor-like cells (HSPCs) derived from human iPSCs. Method: Human cord blood cells and dermal fibroblasts reprogrammed with a lentiviral vector expressing Oct4/Sox2/Klf4/c-Myc. Differentiation of iPSCs into the hepatocytic lineage was induced by serial cytokines stimulation (activin A, fibroblast growth factor, bone morphogenetic protein, and hepatocytes growth factor). Highly proliferative HSPCs from this culture were purified using flow cytometry with antibodies against CD13 and CD133, which are known to be cell surface markers of hepatoblasts in mouse fetal livers. These purified cells were cultured on feeder cells in medium supplemented with growth factors. The CD13+CD133+ cell-derived colonies were characterized by the expression of hepatocytic marker genes (HNF4α and α-fetoprotein) and cholangiocytic marker genes (cytokeratin 7 and 19). HSPC maturation assays were done by inducing spheroid formation and assessing their liver functional gene expression.

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HSPC-derived colonies were serially passaged to determine their proliferative ability. Result: Difference of sources (blood cells and fibroblasts) of iPSCs did not appear to change the efficiency of induced hepatic differentiation. We found that CD13+CD133+ cells from iPSCs-derived hepatic cell cultures have the same potential of HSPCs. Individual HSPCs gave rise to a relatively large colony (contained more than 100 cells) in growth factors-supplemented culture on feeder cells. These colonies expressed both hepatocytic marker genes (HNF4α and α-fetoprotein) and cholangiocytic marker genes (cytokeratin 7 and 19). These HSPCs-derived colonies could continue to proliferate for long periods (over 30 days). After serial passaging, these colonies still contained proliferative cells expressing both HNF4α and Ki-67. Expression of the mature liver function gene CYP3A4 was induced by the spheroid formation of these HSPCs. Conclusion: In this study, we successfully utilized human iPSCs as a source to generate and expand purified HSPCs. These cells displayed similar characteristics to their correspondents *in vivo* including colony forming potential by a single cell and the expression of hepatoblast specific markers. This supports the idea of utilizing this system to determine the molecular mechanisms in regulating liver development. In addition, it is hoped that this in vitro method of liver regeneration may one day replace whole organ transplantations to bypass clinical issues such as organ availability and immune rejection.

Poster Board Number: F-1115

ISOLATION AND CHARACTERIZATION OF ADULT HUMAN LIVER PROGENITOR CELLS

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Adult intrahepatic liver progenitor cells are a facultative stem cell with the ability to differentiate into cholangiocytes or hepatocytes. Developing assays to isolate and characterize these cells is important to studies of chronic liver injury and cell therapy. We generated monoclonal antibodies recognizing cell surface antigens to allow isolation of subtypes of epithelium from dispersed human liver tissue that were unsuitable for orthotropic liver transplantation. Long-term self-renewing liver organoid formation capacity was found in only a subset of epithelial cells defined as CD45-/CD31-/DHIC5-4D9+. Organoid formation efficiency occurred between 6-12% in these cells among the 5 patients samples studied. Single cells formed organoid structures comprising thousands of cells that could be passaged and reinitiate daughter organoid structures in a 3-dimensional matrigel culture system. Organoid formation was specific to the DHIC5-4D9+ population; hepatocytes, endothelium, fibroblasts, and stellate cells were unable to form organoid structures. The more abundant CD45-/CD31-/DHIC5-4D9- /DHIC2-4A10+ duct cells did not initiate organoids, were sensitive to osmotic stress, and likely represent a more mature duct cell. Immunohistochemical analysis confirmed that DHIC5-4D9 and DHIC2-4A10 antibodies localize to distinct cell populations within the biliary system, which co-express duct marker CK19. To assess gene expression, RNA-sequencing libraries were successfully made from as few as 15,000 fresh-sorted cells. The RNA-sequencing profiles of the DHIC5-4D9+ and DHIC2-4A10+ epithelial subsets, and the results of cell transplantation studies into immunodeficient mice will be useful in further characterizing the stem-potential of these cells.

Poster Board Number: F-1116

GENERATION OF FUNCTIONAL TIGHT JUNCTIONS IN DIFFERENTIATED HUMAN HEPATIC PROGENITOR CELLS

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Functional tight junctions (TJs) are vital for mature hepatocytes to perform functions, such as canalicular bile secretion and simultaneous sinusoidal secretion of large quantities of serum proteins into blood. Therefore, generation of TJs in differentiated hepatocytes from human fetal hepatic progenitor cells (hFHPCs) is necessary to understand how junctions contribute to the functional organization of mature hepatocytes. We reported herein a two-stage procedure by which hFHPCs can be differentiated to functional hepatocyte-like cells (HLCs) and required of TJ's structures and functions. Firstly, hFHPCs were treated with hepatocytes growth factor (HGF) for 5 days; secondly, the presence of HGF, oncostatin M and dexamethasone in the culture medium for another 5 days induced efficient hepatic differentiation. After 10 days of *in vitro* maturation, the hFHPCs-derived HLCs exhibited characteristic hepatocyte morphology, expressed hepatocyte markers, including albumin, and cytokeratin 8, and possessed hepatocyte-specific activities, such as p450 metabolism, glycogen storage. Barrier function measured as transepithelial electrical resistance (TER) increased two- to threefold, but paracellular fluxes decreased during the differentiation of hFHPCs. The bile canaliculi (BC)-like structures examined by transmission electron microscope were developed between the HLCs. Tight junction-associated protein ZO-1 was expressed along the BC-like structures. Fluorescein diacetate (FDA) added to the medium was secreted into BC-like structure and accumulated without leakage. Our approach should facilitate to study the mechanism of involved in human hepatocyte differentiation, liver function and the pathogenesis of liver diseases like cholestasis.

Poster Board Number: F-1117

ROLE OF THE POLYCOMB GROUP PROTEIN EZH2 IN THE MURINE HEPATIC STEM/PROGENITOR CELLS

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Regulating the self-renewal is required to maintain the stem cells with the situation that cell proliferation is progressed while cell differentiation is suppressed. Recent studies suggest that polycomb group (PcG) proteins are critical in regulating cell proliferation and cell differentiation related genes. PcG protein Ezh2, histone H3K27 methyltransferase, recruits other PcG proteins and promotes gene silencing. In the present study, we investigated the role of PcG protein Ezh2 in the proliferation and differentiation of the hepatic stem/progenitor cells. We examined the change of frequency of Ezh2 positive cells in the liver development. The frequency of Ezh2 positive cells was high at embryonic day(ED)11.5 and decreased as the developmental stage progress. We also observed a high frequency of AFP, hepatoblast-specific marker, positive cells and BrdU positive cells in ED11.5 mouse liver. To examine Ezh2 regulates the proliferation and differentiation of hepatic stem/progenitor cells, Ezh2 conditional knockout mice (Rosa26::CreER(T2)+/-;Ezh2f/f) were used. Severe inhibition of the hepatogenesis was observed

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in the liver that Ezh2 had been depleted in the early stage of the liver development. The size of Ezh2 depleted liver was smaller than the normal liver, and its cell number was decreased. Immunohistochemical analysis of Ezh2 depletion revealed that the frequency of AFP positive cells was decreased in ck8/18, and the frequency of BrdU positive cell was also decreased at ED13.5. The flow cytometric analysis of c-kit- CD49f+/low CD29+ CD45- Ter119- hepatic stem/progenitor cells at ED13.5 showed that the percentage of fractionated hepatic stem/progenitor cells was decreased in Ezh2 depleted liver. In immunohistochemical analysis of the Ezh2 depleted liver at ED17.5, the frequency of ck7, cholangiocyte-specific marker, positive cells was decreased. Next we induced the depletion of Ezh2 in c-kit- CD49f+/low CD29+ CD45- Ter119- hepatic stem/progenitor cells in single cell-base colony assay. As the result, significantly, suppression of colony formation of the hepatic stem/progenitor cells by Ezh2 depletion was founded. In immunohistochemical analysis, the frequency of albumin, hepatocyte-specific marker, positive cells was increased while the frequency of ck7 positive cells was decreased. These results showed that Ezh2 is essential for the proliferation and differentiation of the hepatic stem/progenitor cells. Our data suggests that Ezh2 is a critical factor for the early liver development, and it may be supported by the expansion of hepatic stem/progenitor cells.

Poster Board Number: F-1118

SELF-RENEWAL VERSUS DIFFERENTIATION AS WELL AS THE LIVER REPOPULATION CAPABILITY OF HUMAN HEPATIC STEM CELLS

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Background: Human mature hepatocytes are critical for basic and applied research, but difficult to manipulate and expand in vitro. Isolated hepatic stem cells which could differentiate towards the hepatic lineage may be one of the alternative sources to generate functional hepatocytes. Hepatic stem cells in murine fetal livers, which possess multilineage differentiation potential and self-renewing capability, have been identified with flow cytometric cell sorting and single cell-based assays in our lab. Methods: Using established procedures, we prospectively isolated and characterized human hepatic stem cells. In the present study, human hepatic epithelial cells were evaluated with flow cytometry combined with single cell-based assays, gene expression, immunocytochemistry, cell phase analysis, and a three-dimensional culture system to support cell differentiation. Results: With flow cytometry, surface marker profiling allowed identification of specific subgroups of cells based on their expression of CD66, CD90 and others. This information could then be used to reclaim discrete cell populations of liver cells. The proliferative capability of sorted cells varied, while the putative stem cells clonally expanded in vitro with high frequency to form large colonies. Multilineage differentiation potential was clearly identified in the single cell-derived colony by means of immunocytochemistry and gene expression analysis using various lineage markers. Furthermore, the putative stem cells were clearly labeled with BrdU at high frequency and maintained a similar level of bipotential differentiation capability even after being subjected to serial cell sorting. These results suggest that the isolated putative stem cells contain significant self-renewal potential. However, when hepatic cells differentiate in vitro, they may partially or entirely lose some functions or capabilities. Use of a novel 3D cell culture system allowed these cells to express higher levels of hepatic cytochrome P450 genes than when a traditional culture was employed. Examples of differentiated cell derived from isolated human hepatic stem cells displaying hepatic functions include ammonia removal, cytochrome P450 activity in vitro and liver repopulation of the toxin receptor mediated cell knockout/SCID mice *in vivo*. Conclusion: Human hepatic stem cells could be prospectively isolated and distinguished from other liver cells. Moreover, they could differentiate into functional hepatocytes under certain culture conditions. Therefore, it may offer a benefit to generate a large number of hepatocytes for clinical application as well as for pharmacology, toxicology and virology.

Poster Board Number: F-1119

CONVERSION OF MOUSE FIBROBLAST TO HEPATOCYTE-LIKE CELLS

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Introduction: Hepatocytes are candidate for liver cell therapy, drug discovery and toxicity assay. Production of functional hepatocyte in culture is a big challenge in this era. Hepatocyte-like cells can be derived from embryonic stem cells and even adult stem cells (mesenchymal stem cells) by treatment with developmental growth factors and chemicals in vitro, although the efficiencies are very low. Using a different strategy, we attempt to transdifferentiate mouse embryonic fibroblast into hepatocyte by appropriate developmental cues after transient induction of the reprogramming factors (Oct4, sox2, klf4, c-myc) as we believe this situation can direct the existing epigenetically unstable cells to cross the stochastic pathways of induced pluripotent stem cells. Material and Method: mouse embryonic fibroblast and limbal derived one were isolated from a 12.5 dps embryo and cultured in DMEM medium containing 10% FBS, 1% L-glutamine and 1% NEAA. After 3rd passage, cells were treated with a combination of retroviral Oct4, Sox2, Klf4 and c-myc for one day in the conventional medium containing small molecule inhibitor of JAK/STAT signaling pathway, then the medium was switched to L15 which supplemented with different approved developmental growth factors including EGF, FGF4, HGF, OSM, dexamethasone, hydrocortisone and human serum albumin as a hepatogenic medium in a period of 8-14 days. Results: Morphological changes during the procedure were considerable as we could see some epithelial colonies formed in the plate from day 10 of induction with a high density of nucleus to cytoplasm. Immunocytoflourescence staining showed these epithelial cells expressed albumin as a gold marker of hepatocyte fate as well as alphafetoprotein. Conventional RT-PCR analysis also revealed albumin and alphafetoprotein during the transdiffernetiation period. Interestingly it seems the cell could store glycogen as a functional characteristic of hepatocyte-like cells by PAS staining. Conclusion: these primary experiments showed us mouse fibroblast could be converted by developmental cues after a short period of epigenetic instability in vitro, which can provide a new available and accessible source for production of hepatocyte-like cells for developmental study, drug screening and even future clinical application. More experimentations should be done to evaluate the functionality of the acquired cells *in vivo* and in vitro as well as demonstrating that the cells do not pass the pluripotency state.

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Poster Board Number: F-1120

A SYNTHETIC NANOFIBRILLAR MATRIX MIMICKING *IN VIVO* CIRCUMSTANCES, PROMOTES HEPATIC DIFFERENTIATION OF MOUSE AND HUMAN ES CELLS.

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Liver is an important organ that performs many complexed functions, including carbohydrate metabolism, protein and lipid metabolism, storage of essential nutrients and biotransformation of drugs. Biotransformation of a drug not only involves detoxification but also their bioactivation, where the metabolite is more toxic than the parent drug molecule. Therefore, drug biotransformation plays an important role in the early stages of the drug discovery process. Many researchers often use primary cultures of hepatocytes for assays, but they are short-lived and cannot be maintained in culture for long term. In addition, there are considerable donor dependent variations. Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of the blastocyst. Lines of evidence have shown that ES cells recapitulate normal developmental processes, and suggest that ES cells provide an attractive source for routine access to large numbers of cells that enable the development of new drug-screening strategies. We previously reported that culturing ES cells on a mesonephric cell line, M15, with the addition of specific growth factors resulted in a selective induction of hepatic differentiation of ES cells at a high efficiency (Shiraki N., et al, 2008). Here, we established a novel hepatic differentiation method using synthetic nanofiber as a cell culture scaffold. We performed endoderm and hepatic differentiation of mouse ES (mES) cells using nanofiber based methods in comparison with several different substrata. Functional assays for hepatocytes, such as albumin secretion, PAS staining, indocyanin green (ICG) uptake and cytochrome P450 activity, were carried out. The results revealed that nanofiber functioned as a substratum that promoted hepatic differentiation of ES cells. Moreover, the ES cell-derived hepatic cells showing ICG uptake ability were maintained for more 100 days on nanofiber. These results indicated that nanofiber not only promoted ES differentiation, but also maintained the function of differentiated hepatic cells. We also checked whether this nanofiber procedure was applicable for human ES cells and human induced pluripotent stem (iPS) cells. Next, to clarify the mechanisms what promote ES cell differentiation in nanofiber system, we are focusing on the function of Rac1, one of Rho family proteins, which regulate actin cytoskeleton. Because the cells cultured on nanofiber are able to exhibit *in vivo* like morphology. These morphological differences from conventional two dimensional (2D) culture plates are associated with several members of cytoskeleton. Moreover, it is reported that murine ES cell can self-renew more rapidly on nanofiber than 2D. This proliferation was accelerated via Rac1 activation on this scaffold. So we checked the effect of Rac1 inhibitor, NSC23766, on ES cells during hepatic differentiation. Inhibition of Rac1 cancelled potentiating effects of nanofiber on every stage of endoderm and hepatic differentiation. Our results indicated that morphological change via Rac1 activation controls cellular differentiation, and motility might not only be the consequence of differentiation, but might also be able to trigger differentiation. In conclusion, the synthetic nanofiber is one of the most promising materials that contribute to tissue engineering and drug delivery applications.

Poster Board Number: F-1121

CHARACTERIZATION OF HEMATOPOIETIC NICHES IN MOUSE FETAL LIVER

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During mouse embryogenesis, fetal liver (FL) functions as a major hematopoietic organ for the expansion of hematopoietic progenitor cells (HPCs) as well as hematopoietic stem cells (HSCs). Especially, vigorous erythropoiesis in a FL is a crucial event for pulmonary respiration after birth. While the characteristics of hematopoietic niche and the responsible cells in adult bone marrow (BM) have been well characterized, those in FL remain poorly understood. We investigated the fetal hepatic cells that support hematopoiesis in FLs and found that hepatoblasts mainly provide the niche for hematopoiesis by secreting multiple hematopoietic cytokines including stem cell factor (SCF) and thrombopoietin (TPO). More intriguingly, we found that submesothelial cells highly express erythropoietin (EPO). While hepatoblasts are found throughout FL, submesothelial cells reside beneath the mesothelium that covers the liver surface. To reveal the role of EPO-expressing submesothelial cells in fetal hematopoiesis, we examined the differentiation status of erythroid cells in FL. It is known that erythroid cells differentiate from CD71+ cells to TER119+CD71- cells via TER119+CD71+ cells. Flow cytometric analysis (FCM) and immunohistochemistry (IHC) using CD71 and TER119 antibodies showed that differentiated erythroid cells seemed to be more abundant in the peripheral region rather than the central part of FL, suggesting that submesothelial cells are involved in erythropoiesis at the periphery of FL. Taken together, our results suggest that hepatoblasts and submesothelial cells constitute distinct niches for fetal hematopoiesis in a region specific manner.

Poster Board Number: F-1122

CONSTRUCTION OF CHIMERIC MICE WITH HUMAN IMMATURED HEPATOCYTES

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Background: Transplantation of human hepatocytes as an alternative tool for the therapy of organ dysfunction in regenerative medicine also attracts pharmaceutical industry due to the great prospects of *in vivo* drug screening and metabolism test. In recent reports, urokinase-type plasminogen activator (uPA) mouse and fumarylacetoacetate hydrolase (Fah)-deficient mouse are applied, however, no chimeric mice liver was completely repopulated by the donor cells. Toxin receptor-mediated conditional cell knockout mice, which imitate natural liver dysfunction through causing acute hepatitis with drug treatment, may resolve this problem. In our study, we firstly constructed the chimera mice with the liver greatly occupied by human donor cells, further provided an efficient *in vivo* differentiate microenvironment for inducing immature liver cells to mature hepatocytes so as to compensate for the deficiency of in vitro differentiation. Finally, we measured the drug metabolism activity after treated the humanized mouse with ketprophen. Methods: After recipients are subject to selectively liver damage by diphtheria toxin treatment and parcel immune system inhibition, donor cells derived from human primary fetal liver are transplanted into recipient mouse; we then analyzed the replacement progress,

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in vivo differentiation and liver reconstruction of donor cells in recipients, human albumin concentration was detected by ELISA, chimeric mouse urine was collected for drug metabolism test after treated with ketprophen for transplanted duration from about 3wk to as long as 16wk. Results: In the recipients, all of the animal livers are succeed to be repopulated by human derived primary fetal liver cells. HE staining and immunohistochemistry vividly exhibited that donor cells which can proliferate well and uniformly replace the resident cells also have the potential of liver reconstruction and hepatocyte differentiation. Moreover, we detect mRNA expression level of human albumin and a variety of human cytochrome P450 (CYP) in chimeric mice, the mRNA level of human albumin as well as human CYP3A4 and CYP2C9 was induced as high as 660507-fold, 226617-fold and 132267-fold at 63 days after cell transplantation compared to the donor cells before transplantation. It showed highly efficient differentiation *in vivo* contrast to the in vitro differentiation. Also, most of the chimeric mice with humanized liver had the potential of human albumin secretion, for some recipients, the albumin concentration reached as high as 1600ng/ml after transplanted for about 56 days. Conclusion: We suggest that our chimeric mice with humanized liver might provide an ideal model to support *in vivo* differentiation and had the potential of drug metabolism activity, which could also take great advantage to human pharmacology such as drug screening and drug to drug interaction test.

Poster Board Number: F-1123

BLOCK HEPATOCELLULAR CARCINOGENESIS OF RATS BY REMOVAL OF PRECANCEROUS CELLS WITH AN ACYCLIC RETINOID

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BACKGROUND & AIMS: Hepatocellular carcinoma (HCC) is a predominantly malignant tumor with poor prognosis, a high rate of recurrence, and no effective early diagnosis and cure. Peretinoin, an acyclic retinoid and a potential chemopreventive drug, has reduced the recurrence rate of HCC in some clinical trials. However, its clinical indication has not been determined due to the poor understanding of its therapeutic target and mechanism. In this study, we aimed to determine the precancerous cell which could contribute to HCC development and to identify the target cells and mechanism by which Peretinoin suppresses HCC. METHODS: Rat model with hepatic oval cells emergence and the process of HCC development responding to 2-Acetylaminofluorene/partial heptectomy treatment was used. And the effect of Peretinoin in liver carcinogenesis process in vivo as well as on flow-cytometrically isolated oval cells in vitro was assessed. RESULTS: Purified CD133+CD44+CD45-EC-cells in rat liver exhibited the properties of oval cells, clonal expansion capability, bi-potential differentiation capability as well as stem cell marker expressions. In vivo results showed that the oval cells and their progenies which mediated liver injury repair might be the originating cells of HCC. Peretinoin inhibited the clonal expansion of isolated oval cells in vitro. Oral administration of Peretinoin diminished oval cell occurrence and suppressed both preneoplastic lesion induction and HCC formation. CONCLUSION: These findings suggest that Peretinoin directly prevent *de novo* carcinogenesis of HCC through inhibiting the expansion of precancerous cells such as oval cells. This may offer clinical benefits for patients with HCC of oval cell origin.

Poster Board Number: F-1124

TOWARD A NEW THERAPY FOR LIVER DISEASE: A NOVEL TISSUE ENGINEERING MODEL USING MOUSE LIVER PROGENITOR CELL SPHEROIDS IN A VASCULARIZED CHAMBER

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BACKGROUND: Liver tissue engineering offers an exciting alternative to transplantation for treating liver failure. We present a novel model based on an amalgamation of emerging concepts, each proven to increase implanted cell survival compared to traditional methods. These are: 1) the mouse vascularized chamber to provide a highly angiogenic protected space *in vivo*, 2) adult mouse liver progenitor cells (LPCs) as a proliferative cell source with bipotential hepato-biliary differentiation, and 3) three-dimensional spheroid cell-culture (cell aggregates), known to promote cell survival and differentiation in vitro and increasingly used for *in vivo* cell-delivery. To evaluate the use of LPCs as spheroids, this study was conducted in two phases. In vitro experiments optimized conditions for generating and maintaining LPC spheroids, and characterized their development over time to select a suitable time-point after formation for spheroids to be used in animal studies. *In vivo* implantation of LPC spheroids in the vascularised chamber then compared this to the traditional seeding of dissociated cells to confirm that the spheroid cell-delivery approach indeed increased cell survival and differentiation. METHODS & RESULTS: In vitro: In methylcellulose media 20,000 cells formed one uniform spheroid per well within 24 hours, providing a rapid, standardized method for culturing and characterizing spheroids. Histology over 10 days (n=6 per time-point) indicated that day 2 spheroids were optimal for implantation, with the highest ratio of proliferation (Ki67 immunolabelling) to apoptosis (caspase). Dissociation of spheroids demonstrated that at day 2 each spheroid contained 22,400 cells. *In vivo*: Bilateral chambers were established on SCID mouse epigastric blood vessels in the groin. Suspended in Matrigel, in one chamber 200,000 dissociated cells were implanted and 9 spheroids (day 2 post-culture) in the opposite chamber, with the same total number of cells in both chambers. Fourteen and 45 day chambers (n=7 each) were evaluated by immunohistochemistry (panCK - LPC marker, and CK18 - hepatocyte marker) and morphometry to determine LPC survival and differentiation. Additionally, CD31 immunolabelling of blood vessels demonstrated that at 14 days capillaries penetrate the spheroids. LPCs (panCK+) increased over time in both spheroid and control chambers, but hepatocytes (CK18+) increased only in spheroid chambers. Significantly increased LPCs ($p<0.05$) and hepatocytes ($p<0.0005$) were found across both time points in spheroid chambers compared to control chambers. CK18+ cells formed hepatic acini indicating tissue (glandular) organisation. No bile ducts were observed. Studies are underway to characterize the functional capacity of this tissue by investigating the activity of HNF4α, a major regulator of hepatocytic differentiation, as well as downstream liver functions governed by this pathway. CONCLUSION: Spheroid cell-delivery significantly increases cell survival and differentiation, compared to dissociated cells. By considerably increasing spheroid numbers being seeded, this spheroid and chamber model offers promise in generating a therapeutically significant mass of liver tis-

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sue for treating liver failure. The extra-hepatic location of this chamber could contain new tissue to ameliorate metabolic liver disease, or alternatively provide a site free of cirrhosis and local disease for the development of replacement tissue for transplantation.

Poster Board Number: F-1125

CELLULAR BASIS OF LIVER REGENERATION AFTER SURGICAL RESECTION

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The liver is a unique organ with a remarkable capacity to regenerate. However, the mechanism of liver regeneration depends on the type of injury. Liver stem cells give rise to two types of liver epithelial cells, i.e., hepatocytes and bile duct epithelial cells. Liver stem/progenitor-like cells, known as oval cells, are believed to repair the injured liver in a certain type of liver injury caused by hepatotoxin or bile duct ligation. By contrast, in regeneration after surgical removal of 70% of the liver mass (partial hepatectomy, PHx), it has been generally accepted that hepatocytes undergo one or two rounds of cell division to regenerate liver. We have reexamined this liver regeneration model by using a genetic tracing system of individual hepatocytes, and found that not all hepatocytes undergo cell division. Analysis using the imaging cytometric system revealed that hepatocytes significantly enlarge prior to cell proliferation. Quantitative analysis indicated that the hypertrophy and proliferation almost equally contribute to the regeneration after 70% PHx, whereas regeneration after 30% PHx is mostly achieved by only hypertrophy. These results indicate that hypertrophy is the first process by which hepatocytes regenerate liver, and the cell division occurs secondly only when hypertrophy is not sufficient. Furthermore, detailed cell cycle analyses of individual hepatocytes during liver regeneration revealed that many hepatocytes do enter into S-phase but do not undergo M-phase, leading to an increase in ploidy. Based on these results, we propose a revised view on the mechanism of liver regeneration after surgical resection.

Poster Board Number: F-1126

ABBRUPTION OF TRANSFORMING GROWTH FACTOR-B1 INDUCED SPONTANEOUS MESENCHYMAL-EPITHELIAL TRANSITION OF RAT ADULT HEPATIC PROGENITORS

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Background: Although epithelial to mesenchymal transition (EMT) contributes to liver tissue repair, it could adversely cause liver fibrosis and promote hepatocellular carcinogenesis. We've learned rat adult hepatic progenitors, which aids in liver regeneration when the proliferation capacity of mature hepatocytes is compromised, could experience an EMT process under the incubation of transforming growth factor (TGF)-β1, which is not only a fibrogenesis cytokine but also a well-known EMT inducer. The aim of the present study is to analyze whether the EMT hepatic progenitors could experience a reverse process of EMT, i.e. mesenchymal to epithelial transition (MET). Material and Methods: Hepatic oval cells were isolated from rats fed a choline-deficient diet supplemented with ethionine and characterized by flow cytometry. For EMT inducement, the cells were cultured in 10% FBS-DMEM/F12 medium containing 1ng/ml TGF-β1 for 16 days with the medium replaced every 2 days. For MET inducement, the medium were changed to 10% FBS-DMEM/F12 supplemented with 1ng/ml epidermal growth factor and 0.5ng/ml stem cell factor for 7 days, with the medium replaced every 2 days. Results: Hepatic oval cells were positive for the progenitor cell-specific markers OV-6, α-fetoprotein and Dlk, as well as the hepatocyte markers albumin and the cholangiocyte markers cytokeratin 19. In the presence of TGF-β1 for 16 days, cell proliferation was inhibited and the cells flattened down with the cell size increased about 3 to 4 times larger at the 16th day than that at 0 day. Real-time PCR results showed TGF-β1 upregulated snail expression with 3 times and kept 20% downregulation of e-cadherin at the 16th day if compared with 0 day, indicating hepatic oval cells experienced a sustained EMT process. During this process, there was a time-dependent increase of extracellular matrix, including collagen I, collagen III, connective tissue growth factor, tissue inhibitor of matrix mellatoproteinase. During the seven days abruption of TGF-β1, the EMT cell size gradually restored to the size of the control cells. Real-time PCR results showed that e-cadherin expression increased more than twenty times at the 1st day, while the expression of snail reduced to 0.4% at 7th day of that at 0 day, and the expression of the extracellular matrix related genes reduced to about 20% at 7th day of that at 0 day, indicating a MET reversion process. Furthermore, the MET cells showed similar growth rate with the control cells. In addition, although the expression of a-smooth muscle actin, a myofibroblast marker, increased during EMT and reduced during MET, the expression of the progenitor cell marker, α-fetoprotein and Dlk, changed little during all the EMT/MET process. Conclusion: Abbruption of TGF-β1 induces spontaneous MET of rat adult hepatic progenitors, indicating removal of the underlying injurious stimulus and reduced tissue TGF-β1 level might contribute to hepatic progenitor-related liver regeneration.

Poster Board Number: F-1127

REPROGRAMMING OF TRANSFORMED HEPARG HEPATOCYTES INTO BIPOTENT PROGENITORS

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Cancer stem, or progenitor, cells have been identified in hepatocellular carcinoma (HCC). Therefore, a subtype of HCC histologically characterized by carcinomas of combined type (combined hepatocellular and cholangiocarcinoma) and by the expression of "stemness"-related markers have been proposed. One of the main difficulties to characterize hepatic cancer stem cells is the absence of experimental models representative of these cells. In this context, the HepaRG cell line has retained the capacity to differentiate into hepatocytes and biliary cells and is therefore considered as the only cell line of human hepatic progenitors. In addition, HepaRG hepatocyte-like cells isolated from differentiated cultures are able to transdifferentiate through hepatic progenitors into hepatocyte- and biliary-like cells (Cerec et al., 2007). The aim of our study was to identify the mechanisms involved in the reprogramming of HepaRG hepatocytes in progenitor cells. A transcriptomic analysis has been performed with GeneChip Human Genome U133Av2 Array (Affimetrix). Gene expression profiles of mature HepaRG hepatocytes and cells 1, 4, 8, 12, 16, 24 and 48 hours after selective detachment and seeding were analyzed. We demonstrated that expression of the transcription factor, SNAI2, increases early in the reversion process. This up-regulation was associated with the repression of its target gene, E-cadherin (CDH1), and correlated with the disappearance of the E-cadherin protein after cell seeding. All these changes suggested that the reversion of HepaRG-hepatocytes into progenitors might result from an epithelial-mesenchymal-transition (EMT) process. By using a Gene Set Enrichment Analysis approach, we further showed that molecular profiles data at 1 and 4 hours showed an enrichment

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of the TGFβ signature at 4h of reprogramming. Interestingly, this signature is also related to bad prognosis in HCC. We also demonstrated a stem cell signature at 48h (reprogrammed cells) compared with differentiated HepaRG-hepatocytes. Presence of stem cell markers (Oct4, Nanog) in reprogrammed HepaRG cells was also confirmed by immunocytochemistry. Altogether, the HepaRG cell line represents an interesting model to study mechanisms involved in mature hepatocyte reprogramming into bipotent progenitors expressing various stemness markers. Furthermore, this reprogramming resulted from an EMT process. [Acknowledgement] Part of this research was supported by the INSERM/Japan Society for the Promotion of Science (JSPS) cooperation program.

Poster Board Number: F-1128

G1 CELL CYCLE CHECKPOINT ESTABLISHMENT AND ASSOCIATED DNA REPAIR KINETICS *IN VIVO* DURING EMBRYONIC DEVELOPMENT

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All organisms are consistently exposed to endogenous and exogenous stress that causes lifelong DNA damage; thus, eukaryotic cells have developed cell cycle checkpoints and DNA repair mechanisms that act in an orchestrated manner to arrest the cell cycle until damage is repaired. If DNA damage cannot be repaired, then one of the consequences is cancer. Embryonic stem (ES) and embryonic carcinoma (EC) cells are defective in G1 checkpoint after DNA damage. Differentiated EC cells show an increased G1 cell population but still lack of G1 checkpoint, even though DNA damage response (DDR) proteins activation appears to be normal. Thus, at which development stage and under what circumstances is the G1 cell cycle checkpoint necessary for a cell is still unclear. We investigated DNA damage checkpoint and repair kinetics *in vivo* in mice embryonic liver cells at different developmental stages. Pregnant ICR mice were exposed to ionizing radiation (IR), embryonic liver cells at E11.5, E13.5, E15.5, and E17.5 showed transient G2 cell cycle arrest. Starting from E13.5/E15.5, there was a significant G1 arrest, which was absent in E11.5 liver cells. After IR, the G1 checkpoint regulator p21 was enhanced to a greater level at E15.5 compared to E11.5, which was accompanied by reduction of CDK2 and CDK2/Cylin E complex. Other cyclins and CDKs did not display apparent periodicity in accordance with cell cycle progression. Therefore, the establishment of the G1 checkpoint at E15.5 might be regulated by p21 mediated down regulation of CDK2/Cylin E. Although it has been shown that ES cells have a higher DNA repair capacity than their differentiated derivatives, we found that at certain embryonic stage there was a turnover of DNA damage induced repair mechanisms; such as robust enhancement of repair protein and repair activity at E15.5 compared to at E11.5. As a result, IR induced double strand break foci (γH2AX foci) were removed (repaired) more efficiently in E15.5 than in E11.5 liver cells; more importantly, number of chromosomes with breakage and rearrangements in adult liver cells were significantly less detected when IR treating mice at E15.5 than at E11.5. Moreover, our preliminary data showed that E15.5 could be a stage of epithelial to mesenchymal transition with mesenchymal proteins started to express in embryonic liver cells. Taken together, this is the first *in vivo* study of cell cycle checkpoint during embryonic development. We found that E15.5 is a critical stage for the establishment of DNA damage mediated G1 checkpoint and magnification of DNA repair mechanism, which might have important biological impact on organogenesis, genome integrity.

Poster Board Number: F-1129

RNA-SEQ PROFILING OF SINGLE CELLS DURING IN-VITRO HEPATIC DIFFERENTIATION

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Directed differentiation of pluripotent stem cells (PSCs) into specific cell types in-vitro begins with a relatively homogenous population of undifferentiated cells. Upon addition of growth factors, PSCs are capable differentiating towards specific cell types of interest, however, the outcome of differentiation often results in low efficiencies and heterogenous cell populations. In addition to improving differentiation yields, understanding the different trajectories of single cells during in-vitro differentiation, under seemingly uniform conditions, can help understand the transcriptional regulatory networks required to specify and maintain cellular fate. Using an established hepatic differentiation protocol, we employed RNA sequencing (RNA-Seq) to obtain single base resolution of single cells during a hepatic differentiation from human ESCs. During the initial phase of data generation, we collected eight single cells from three critical phases of hepatic differentiation: undifferentiated hESCs, definitive endoderm (day 5), and hepatic progenitor cells (day 8). In all cells, lineage-specific markers representative of each differentiation phase were detected. Despite this, principle component analysis (PCA) clustering results suggests less homogeneity among cells as differentiation progressed where hepatic progenitor cells were the least cohesive. Inspection of transcriptional regulatory factors (TF's) in hepatic-like cells using in-silico TF motif predictions of distal promoters among differentially expressed genes suggests combinatorial use of endodermal/hepatic progenitor TF's. These initial results highlight that lineage markers alone are not sufficient to characterize differentiation and that molecular characterization can highlight critical differences that can help predict cellular trajectories from a parental source. This study demonstrates the application of next generation sequencing technology at single cell resolution to understand in-vitro differentiation and ultimately cell fate decisions.

Poster Board Number: F-1130

ESTABLISHMENT OF NOVEL DETECTION SYSTEM FOR EMBRYONIC STEM CELL-DERIVED HEPATOCYTE-LIKE CELLS BASED ON NONGENETIC MANIPULATION WITH INDOCYANINE GREEN

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Hepatocytes derived from embryonic stem cells (ESCs) are expected to be useful for basic research and clinical applications. However, in several studies, genetic methods used to detect and obtain them are difficult and pose major safety problems. Therefore, in this study, we established a novel detection system for hepatocytes using indocyanine green (ICG), which is selectively taken up by hepatocytes, based on nongenetic manipulation. ICG has maximum light absorption near 780 nm, and it fluoresces between 800-900 nm. Making use of these properties, we developed flow cytometry

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equipped with exitation laser of 785 nm and specific bandpass filters and successfully detected ESC-derived ICG-positive cells that were periodic acid-Schiff positive and expressed hepatocyte phenotypic mRNAs. These results demonstrate that this detection system based on nongenetic manipulation with ICG will lead to isolate hepatocytes generated from ESCs and provide the appropriate levels of stability, quality and safety required for cell source for cell-based therapy and pharmaceutical studies such as toxicology.

Pancreatic Cells

Poster Board Number: F-1131

STEMDIFF™ DEFINITIVE ENDODERM: A FULLY DEFINED ANIMAL COMPONENT-FREE MEDIUM FOR EFFICIENT DIFFERENTIATION OF HUMAN PLURIPOTENT STEM CELLS TO DEFINITIVE ENDODERM.

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The formation of definitive endoderm (DE) from human pluripotent stem cells (hPCSs) is a required intermediate step in the development of more specialized cell types of endoderm organs including the pancreas and liver. Following the ground-breaking studies by Kevin D'Amour and colleagues (*Nat. Biotech*., 2006) showing that Activin A can induce expression of key DE markers, significant progress has been made in the development of robust protocols for producing DE from hPSC cultures. A key limitation of this published protocol, however, is the difficulty of reproducing results with multiple available hPSC lines in different labs. While the root cause of reduced performance in different hPSC lines is largely unknown, the use of less defined components including fetal bovine serum may contribute to this variability. Furthermore, the use of animal-derived components in the differentiation medium may ultimately complicate the translation of these protocols into clinical use. Therefore, as a first step towards improving consistency of DE formation from hPSCs, we sought to develop an animal component-free DE differentiation medium that allows for robust performance in multiple human embryonic and induced pluripotent stem cell lines. hES (H1 and H9) or hiPS (4D1) cells were maintained under defined and feeder-free conditions on Matrigel™ in mTeSR™1 or TeSR2™. Clump cultures were enzymatically dissociated and re-plated as single cells at high density ($2x10^5$ cells/cm^2) in the presence of Y-27632 and cultured overnight prior to differentiation. The following day, cells were treated with STEMdiff™ Definitive Endoderm basal medium plus supplements for a further 72 hours then analyzed for expression of key DE markers CXCR4 and SOX17 by flow cytometry. Our results showed that 81.8 ± 2.3 % (mean ± SE; n = 14) of H9 cells, 90.4 ± 1.9 % (n = 3) of H1 cells, and 71.6 ± 5.4 % (n = 10) of 4D1 hiPS cells co-expressed CXCR4 and SOX17 following 72 hours of culture. Next, using published protocols, we further demonstrated the ability of this DE cell population to differentiate towards pancreatic (Rezania et al., *Diabetes*, 2011) and hepatic (Hay et al., *PNAS*, 2008) lineages as assessed by immunocytochemistry for PDX-1 and human serum albumin (HSA), respectively, indicating that the definitive endoderm-enriched population formed using this protocol maintains potency towards multiple endoderm tissue types. We have developed a fully defined, serum- and animal component-free media formulation (STEMdiff™ Definitive Endoderm) that provides rapid and efficient differentiation of hPSCs to DE. Cultures differentiated using this new media demonstrated robust DE marker expression and maintained the ability to be directed towards pancreatic or hepatic lineages. The protocols described here can be reliably used as the starting point for studies aimed at the formation of endoderm cell types from hPSCs. Future work will focus on optimization of these protocols with defined matrices for efficient DE formation.

Poster Board Number: F-1132

DIFFERENTIATION OF HUMAN IPS CELLS INTO PANCREATIC LINEAGES IN XENO-FREE CHEMICALLY DEFINED CULTURE SYSTEMS

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Human ES/iPS cells have the potential to differentiate into all major lineages of somatic cells. One possible application of human ES/iPS cells is to generate pancreatic β-cells for clinical application in the treatment of Type-I diabetes. Several groups have reported differentiation of human ES/iPS cells into pancreatic-endocrine cells in various *in vitro* feeder-cell culture systems (Chen et al., 2009; Kroon et at., 2008; Cho, et al., 2008; DAmour et al., 2006) or feeder-free culture systems using matrigel, gelatin or purified ECM protein coatings (Thatava et al., 2010; Zhang et al., 2009; Mao et al., 2009; Eshpeter et al., 2008; Jiang et al., 2007). But, these differentiation methods have some limitations, such as limited efficiency to generate functional pancreatic β-cells, use of chemically-undefined raw materials, risk of xenogenic contamination, and use of various biological coatings which often show high batch-to-batch variability. But, widespread clinical application of hES/iPS cells-derived pancreatic cells will require culture methods that are robust, reproducible, xeno-free, and use chemically-defined raw materials. To overcome the problems-associated with the available techniques, we are now trying to establish a method for differentiation of human iPS cells into insulin-secreting pancreatic β-cells using a novel cell culture scaffold with xeno-free synthetic surface. We have explored a stepwise protocol using feeder-free culture condition along with chemically defined raw materials to differentiate human iPS cells into pancreatic lineages by mimicking major signals of human embryonic pancreas development. We performed RT-PCR and Immunostaining at different stages of differentiation to check the expression pattern of the marker genes and proteins, respectively, in the differentiated cells. Our results showed that human iPS cells successfully differentiated into SOX17+ definitive endoderm cells (56%) at day-5, HNF4α+ primitive gut tube cells (36%) at day-8, and PDX1+ pancreatic progenitor cells (32%) at day-14. Results also confirmed that a large proportion of PDX1+ progenitor cells further differentiated into INSULIN+ pancreatic β-like cells at day-28. But, these INSULIN+ cells co-stained with GLUCAGON (α-cell) and some of them also co-stained with SOMATOSTATIN (δ-cell), indicating immature pancreatic endocrine cells. Although a good percentage of cells differentiated into PDX1+ pancreatic progenitor cells at day-14, they were not PTF1A+ at this time point of differentiation. Therefore, we are now optimizing our culture condition to enhance differentiation efficiency and to identify the exact time point of differentiation into PDX1+/PTF1A+ pancreatic progenitor cells, and also into NGN3+ endocrine progenitor cells. Our ultimate goal is to generate functional pancreatic β-cells in xeno-free and chemically defined culture system, which may open a new avenue for the use of cell therapy in diabetes in the near future.

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Poster Withdrawn

Poster Board Number: F-1134

DIFFERENTIATION OF RAPAMYCIN PRIMED-HUMAN EMBRYONIC STEM CELLS INTO PANCREATIC PROGENITOR CELLS

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Pancreas is an important endodermally derived organ and because of some serious related diseases, like type I diabetes, production of different pancreatic cell types from pluripotent cell sources is of major importance in regenerative medicine. According to our previous report for differentiation of human embryonic stem cells (hESCs) to definitive endoderm (DE), from 20 different combinations of small molecules and activin A, rapamycin (100 nM, Rapa, as the priming inducer) and activin A (50 ng/ml, Act50, as the differentiation inducer) were selected. In this study, the competency of two DE populations which derived from Rapa-Act50 and Wnt/Act100-Act100 inductions were evaluated for pancreatic progenitors (PP) differentiation according to five published protocols. By quantitative real time RT-PCR and immunofluorescence staining a significant difference was observed in expression of *HLXb9*, *HHEX*, *HNF4a*, *ISL1*, *NEUROD1*, *NKX6-1*, *PAX4*, *PTF1a*, *PDX1*, *NGN3* genes between studied PP differentiation protocols, while these differences were not significant between Rapa-Act50 and Wnt/Act100-Act100 groups. Therefore, DE cells produced from Rapa-Act50 induction have the same PP differentiation competency comparing to the DE cells produced from Wnt/Act100-Act100 induction. Additionally, the protocol type influenced the PP differentiation efficiency of similar DEs. This study can help in developing new, more defined differentiation methods, and new signaling pathways for pancreatic differentiation.

Poster Board Number: F-1135

A STEP WISE APPROACH FOR THE SCREENING OF LOW MOLECULAR COMPOUNDS THAT INDUCE PANCREATIC BETA CELL FROM INS1/ GFP MES CELLS.

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Loss of beta cells or functional defects results in improper glucose homeostasis, which leads to type 1 or type 2 diabetes. In type 1 diabetes (T1D), beta cells are almost completely lost as result of a destructive autoimmune process. To cure T1D, cell replacement therapy is effective as it is caused by the lack of a single, well-defined cell type, the beta cell. Stepwise differentiation from embryonic stem cells (ESCs) to functional insulin-secreting pancreatic beta cells may yet prove useful for cell replacement therapy for T1D.Though several protocols have been reported to directly form insulin-producing cells from ESCs, these protocols produce few cells with demonstrable beta-cell phenotypes at the end of the procedure. Screenings for compounds that induce the differentiation into definitive endoderm (Zhu *et al*., 2009) or pancreatic progenitor (Chen *et al*., 2009) were reported. Our group (Sakano *et al,*) have screened compounds that induce beta cells from pancreatic progenitor, and identified drugs that involved in beta-cell differentiation (Sakano *et al*.,unpublished). Here, we have newly designed a three-step differentiation protocol of mouse ESC towards pancreatic beta cells. Step 1: differentiating ESC into definitive endoderm .Step 2: differentiating definitive endoderm into pancreatic progenitor. Step 3: differentiating pancreatic progenitor into pancreatic beta cell. In our present pancreatic beta cell differentiation protocol, a mouse ESC *Ins1*/ GFP cell line, which enables us to quantify the number of *Ins1*/ GFP positive cells is used. Then we screened for compounds that worked synergistic with the above Sakano's hit compounds to induce pancreatic progenitor or beta cells at step 2 or step 3. Now the screening is on going. After getting several candidate compounds, we will exam dose dependencies and glucose responsiveness of the resultant differentiated cells.

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Poster Board Number: F-1136

A NEONATAL MOUSE MODEL OF PANCREATIC BETA-CELL REGENERATION AFTER DESTRUCTION BY STREPTOZOTOCIN

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Renewal of beta-cells postnatally originates from several sources. Identifying the molecular mechanism of pancreatic beta-cells regeneration might lead to a potential treatment to cure diabetes mellitus. Here, we aim to establish a pancreatic regeneration model using streptozotocin (STZ), a drug which is known to destroy beta-cells and elucidate the molecular mechanism underlying the regeneration process. In the newborn rat, it is reported that spontaneous beta-cell regeneration does occur after STZ administration at subdiabetogenic dose. The damage caused by STZ is followed by the recovery of insulin-producing cells, and it is reported that alpha-cell hyperplasia was observed within preexisting islets. In the mice, STZ induced beta-cell destruction model is a frequently used model for the assessment of drugs that protect beta-cells from apoptosis or rescue hyperglycemia. Although many studies employed high does of STZ and focused on the acute phase of the beta-cell destruction and on the expression of differentiation markers during hyperglycemia, there are very few reports showing the reversal of blood glucose. In this study, we sought to establish a condition that reversal to normoglycemia occurred after hyperglycermia caused by STZ mediated beta-cell destruction. We investigated the dose dependent diabetogenic action of STZ administration in neonatal mice at postnatal day 7 and measured the blood glucose up to 150 days. We found that doses at above 110 mg/kg STZ elicited slow progressive diabetes and no reversal of blood glucose after long term observation. However, at doses of 50 mg/kg STZ, the reversal of blood glucose was observed, particularly at a high rate in the female mice. We then performed glucose tolerant test, and stained the islets with anti-insulin or anti-glucagon antibodies at different time points after STZ-treatment to quantify the numbers of islet, sizes of islets and insulin positive areas. The insulin positive cell number and islet sizes dramatically decreased on day 50 after STZ treatment. The number of beta-cells in the islet partially recovered by 150 days after STZ treatment. These results indicate that a regeneration of beta-cells occurred. To investigate the mechanism of the regeneration of beta-cells, we performed immunohistochemical analysis and tried to define the cell source for the recovery of beta-cell.

Poster Board Number: F-1137

TRANSDIFFERENTIATION OF TYPE I DIABETES PATIENTS SKIN FIBROBLASTS INTO ISLET-LIKE CLUSTERS BY PURE CHEMICAL INDUCTION

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Cellular differentiation has previously been considered an irreversible process in adult mammalian cells. However, the terminal differentiated state of cells is not permanent and can be altered, and the expression of silent genes can be induced. The aim of our work was to induce pancreatic islet gene expression into human skin fibroblasts from patients with type I diabetes through purely chemical agents. For this goal a 3-mm diameter of skin biopsy was extracted from two patients with type I diabetes and one healthy volunteer using a sterile biopsy punch. Protocols and informed consent were approved by the Committee of Ethics of Investigation Protocols from Hospital Italiano de Buenos Aires. The biopsies were cultured in DMEM-Knockout supplemented with 10% FBS and 1% of antibiotic at 37°C in 5% CO_2. Then 1.5 x10^5 human fibroblasts cells were seeded per well of a six well plate in DMEM/F12-Knockout medium with 40 ng/mL bFGF, 20% Xeno Free Serum replacement, 2 mM glutamine, 0.1 mM nonessential amino acids, 0.1 mM β-mercaptoethanol, 2% antibiotics. A sequential multistep differentiation protocol with different grown factors and chemical agents including IGF1, Nicotinamide, Exendin-4, Insulin-Transferrine-Selenium, B27 supplement and glucose was performed during 30 days. A group of cells from each patient and control donors were replated onto CellsStar (Invitrogen) coated 6-wells dishes during the 7 final days of treatment. The pancreatic gene expression study by RT-PCR, glucagon immunocytochemical analysis and the chromosomal abnormality were assessed in the treated cells. All treated fibroblasts showed islet-like cluster morphology and the expression of PDX1, NGN3, GLUT2, somatostatin and glucagon genes was detected after chemical treatment; in addition they were positive for glucagon immunocytochemical analysis. Only when the cells were cultured in gelatin substrate, insulin gene expression was found in one patient with type I diabetes. To demonstrate the transdifferentiation nature of our protocol, (without passing through pluripotent state) at day 15 of the treatment we transfected the treated cells with GOF18-EGFP plasmid that contains GFP under OCT4 promoter control. As a result no-expressing GFP cells were found. G-banding of genomic DNA revealed a normal karyotype after the chemical treatments. At the end of the treatment, a group of islet-like clusters from one type I diabetes patient were cultured by another 30 days in DMEM-Knockout with 10% SFB and antibiotics, replacing the medium once a week. Surprisingly, these cells returned to the fibroblasts morphology and were negative for glucagon and somatostatin by RT-PCR. New studies will be necessary to confirm, if these cells went back to the fibroblasts state or some remaining fibroblasts grow till colonize the cell culture dish. Here we demonstrate for the first time that human skin fibroblasts from type I diabetes patient could be reprogrammed to islet-like clusters that contain insulin, glucagon and somatostatin expressing cells through transdifferentiation without using transgenic strategies. This novel approach confirms the amazing cellular plasticity, opening great possibilities in the future of the clinical application of regenerative medicine in Diabetes patients.

Poster Board Number: F-1138

IDENTIFICATION OF PANCREATIC STEM / PROGENITOR CELLS EXPRESSING PDX1 RESIDE IN THE CD133 POSITIVE PANCREATIC DUCT

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The existence of progenitor cells in the adult pancreas has been a long-standing topic of debate. Here, we provide direct evidence for the first time that the Pdx1+ cells residing in the pancreatic ducts show the characteristics of facultative multipotpent pancreatic progenitor cells. Pdx1+ cells persist from fetal to adult mice and we observed enhanced proliferation of Pdx1+ cells in a partial ductal ligation adult mouse model. Flow-cytometric sorting of Pdx1+ cells further showed that the Pdx1-DsRed2dimSSCmid fraction includes cells with clonal expansion and multi-lineage differentiation capacities. Moreover, these Pdx1+ progenitor cells exhibited asymmetric cell division, a known characteristic of stem/ progenitor cells. We

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also succeeded in quantifying the balance between asymmetric and symmetric cell division in Pdx1+ progenitor cells. Our current results thus indicate that the Pdx1+ cell populations in the pancreatic ducts include pancreatic progenitor cells with clonal expansion and multipotency and that undergo asymmetric cell division.

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SENSORY NEURONS CONTROL PANCREATIC B-CELL REGENERATION

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Pancreatic islets are heavily innervated by dorsal root ganglia sensory neurons. Previous studies revealed the involvement of different sensory afferents in β-cell function and glucose homeostasis. Moreover, genetic abnormality in C-fiber neurons of NOD mouse model is associated with type 1 diabetes. Here we investigate the interaction between β-cells and sensory neurons which leads to β-cell regeneration. Our findings show that sensory neuron have a strong tropism for β-cells when co-cultured .Confocal and electron microscopy imaging also exhibited the ability of the two cell types in forming synaptic connections. In the presence of sensory neurons, β-cell proliferation and the number of pancreatic stem cells are enhanced. After exposure to dorsal root ganglion (DRG) neurons, clonal pancreatic stem cell colonies produce significantly more β-cells upon differentiation. We also show that diabetic conditions alter the interaction of β-cells and sensory neurons by affecting both cell types. Diabetic β-cell fail to attract normal sensory neurons and diabetic sensory neurons are less attracted to normal β-cells. These findings suggest an important the role for sensory neurons in induction of β-cell regeneration under normal and diabetic conditions.

Poster Board Number: F-1140

GLUCOCORTICOIDS MODULATE ACINAR-TO-DUCTAL TRANSDIFFERENTIATION IN PANCREATITIS

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Acinar-to-ductal or acinar-to-hepatic metaplasia has been considered as either an initial event or risk factors for development of pancreatic neoplasm which is believed to be mediated by initial dedifferentiation of mature exocrine cells to generate a population of nestin- or ABCG2-positive intermediate precursors. However, the molecular mechanism controlling the process is less clear. Recent reports demonstrated that acinar cells can directly transdifferentiate into ductal-like cell during chronic pancreatitis. We therefore hypothesize that certain inflammation-induced factors may responsible for induction of acinar-to-ductal transdifferentiation. Initial analysis identified that activated glucocorticoid receptor was expressed in patients of chronic pancreatitis and in the pancreas of mice that treated with pancreatitis inducer-caerulein. Moreover, we also found glucocorticoid treatment[[Unsupported Character - ]] could enhance the reprogramming of primary mouse acinar cells and exocrine AR42J cell lines to fetal cell-like status as judged by expression of Pdx-1, Ptf1a, Hes1 and ABCG2 in suspension cultures. Indeed, glucocorticoids are known to play an important role in pancreatic cell fate determination and in modulation of inflammatory responses. Moreover, we revealed the level of ductal markers such as DBA and CK19 were increased in the pancreatitis mice. Lineage tracing experiments using Elas-CreER; UBC-floxedDsRedT3-emGFP mice demonstrated that reprogramming of acinar cells to ductal cells represented a true transdifferentiation event. To further determine the role of glucocorticoid receptor, Elas-CreER;GR $^{flox/flox}$ mice was utilized and confirmed that c-Src and Ras activation mediated by activated glucocorticoid receptor was a key regulator of acinar-to-ductal transdifferentiation. Indeed, pancreatitis is a risk factor of pancreatic cancer, and our findings suggest that activation of glucocorticoid pathways in chronic pancreatitis maybe a key step of ductal reprogramming which possibly leads to generation of ductal lesion in pancreatic carcinogenesis.

Poster Board Number: F-1141

E42 PIG EMBRYONIC PANCREATIC TISSUE FOR THE TREATMENT OF DIABETES: PRECLINICAL STUDIES IN NON-HUMAN PRIMATES

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Transplantation of human or pig embryonic tissues offers new possibilities to address the shortage of organs for transplantation. Recently, we demonstrated in mice and NHP proof of concept for the correction of diabetes by E42 pig embryonic tissue. Thus, implantation in omental pockets could induce insulin independence in two Cynomolgus monkeys following induction of diabetes with streptozotocin. Importantly, the growing tissue was found to be predominantly vascularized with host blood vessels, thereby evading hyperacute or acute rejection, which could potentially be mediated by pre-existing anti-pig antibodies. Thus, the vigorous immune rejection associated with xenotransplants was shown to be circumvented upon transplantation of early pig embryonic precursor tissue, with full physiological control of blood glucose levels. Maintenance of the graft during a follow-up period of 8-12 months was attained with conventional immune suppression similar to that currently used in allogeneic islet transplantation. Considering mouse data suggesting a reduced immununogenicity for E42 pig pancreatic tissue compared to tissues harvested at later time points, further efforts to 'fine tune' our immune suppression protocol are in progress. Another attractive option for further improvement is to use a subcutaneous site for implantation instead of the omental site. To that end we conducted experiments in two non-diabetic immunosuppressed NHP. In the first experiment pig embryonic pieces were injected by fine needle under the subcutaneous space. Although some growth of implanted embryonic tissue could be detected, further improvement could be attained in the next animal upon implantation in the subcutaneous tissue under direct vision, through a one cm long incision, thus creating a subcutaneous pocket to host the grafted implants. This animal exhibited robust graft growth and presence of islets which were clearly dividing based on double staining for insulin and Ki67. Similarly to implants in the omentum in previous studies, islets in the subcutaneous sites were predominantly vascularized by host blood vessels. Morphometric analysis revealed an islet mass comparable to that found in the omental graft (4mm^3 for subcutaneous implant and 4.7mm^3 for the omental site). A second question relevant to the clinical translation of our approach is whether the embryonic pig tissue can survive overnight shipping to distant medical centers. Importantly, the quality of pig embryonic tissue in two separate experiments remained unchanged after 28 hrs of cold ischemia. Thus, when pig E42 pancreatic tissue was kept in medium for 28 hrs and implanted thereafter under the renal capsule of a SCID mouse,

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graft size 16 weeks after implantation was similar to that found with fresh tissue (2.95±0.54cm^3 and 2.92±0.45cm^3, respectively). Likewise, the ability to secrete insulin at 12 weeks after implantation, measured by specific ELISA for pig insulin blood levels, was not significantly different (20.22± 16.01ulU/ml and 15.36 ±11.24 ulU/ml, respectively; ($p<0.39$)). In conclusion, correction of diabetes by implantation of E42 pig embryonic tissue in the omentum of NHP is feasible. Moreover, this tissue exhibits marked resistance to ischemia and can be shipped over night to distant medical centers. Studies in diabetic NHP to further explore the curative potential of subcutaneous implantation ,which is less invasive and enabling better monitoring of the implant growth and development, are warranted.

Poster Board Number: F-1142

EFFECTS OF CARBON ION BEAMS ON LIVER CANCER STEM-LIKE CELLS AND ITS COMPARISON WITH X-RAYS

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To investigate whether a carbon ion beam has beneficial effects compared to X-rays by targeting putative cancer stem cells, human liver cancer stem-like cells sorted from HepG2 and Huh7 cells were treated with or without carbon ion or X-ray irradiation and then colony formation assay, spheroid formation assay, *in vivo* tumorigenesis analyses, FACS analysis, as well as gammaH2AX foci formation assay were performed. FACS analysis showed that the proportion of CD133+/CD90+, CD44+/ESA+ was more significantly enriched by X-rays compared to carbon-ion beam. CD133+/CD90+ and CD44+/ESA+ cells significantly have higher number of colony and spheroid compared to CD133-/CD90- and CD44-/ESA- cells. In addition, CD133+/CD90+ and CD44+/ESA+ cells have shown more potential to form tumors in SCID mice than CD133-/CD90- and CD44-/ESA- cells. Colony assay showed that CD133+/CD90+ and CD44+/ESA+ cells appeared to be radioresistant to both X-ray and carbon ion beam, but carbon beam was more effective at killing those of cancer stem-like cells. The number of gammaH2AX foci in CD44-/CD24- cells was higher than that of CD44+/CD24+ cells after irradiation with either X-ray or carbon ion beam. The number of gammaH2AX foci in CD44+/CD24+ cells was almost the same in the early time, but it persists for significantly longer in carbon ion beam irradiated cells compared to X-rays. In conclusion, CD133+/CD90+ and CD44+/ESA+ cells are putative liver cancer stem cells, and carbon ion beams induce prolonged DNA damage, therefore potentially have advantage by targeting liver cancer stem-like cells compared to X-rays.

Intestinal/Gut Cells

Poster Board Number: F-1143

MODELLING THE SPATIO-TEMPORAL DYNAMICS OF MOUSE SMALL INTESTINAL STEM CELLS

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We have modelled the spatio-temporal dynamics of stem cells in the mouse small intestinal crypt as a function of Wnt and Notch signalling generated locally by the neighbouring cells. The highest level of Wnt activity is associated with equipotent stem cells (SCs) and Paneth cells intermingling at the crypt bottom while Notch signalling is activated in cells that are in contact with secretory cells. Newly generated cells reacting to the highest levels of Wnt stimuli will be common goblet-Paneth cell progenitors or SCs. The hypothesis that a newly generated common goblet-Paneth cell progenitors requires to be in contact with more than 3 SCs provides the best fit to experimental observations. The counterpart of this hypothesis is that a newly generated cell will be a stem cell if in contact with more than 3 goblet-Paneth cell progenitors or matured Paneth cells. The physical contact requirement, which is characteristic of Notch signalling and the recently observed activation of Notch1 receptors in intestinal stem cells all indicate that Notch signalling underlies the fate of SCs descendants in the crypt bottom. In our simulated crypt when the number of Paneth cells decreases due to the deletion of the transcriptional factors Sox9, Gfi1 or Math 1, there is a decrease in Wnt signalling that is accompanied of a decrease in the predicted number of stem cells which colocalized with Paneth cells as experimentally observed. The common goblet-Paneth cell progenitors under high Wnt signalling will differentiate into Paneth cells while those migrating out from the crypt bottom will differentiate into goblet cells. We assumed that mature Paneth cells migrating up the crypt bottom undergo anoikis mediated by Eph/ephrin signalling. Eph/ephrin signalling regulates the formation of E-cadherin-based adhesions being these adhesions absent between EphB- and ephrin-B-expressing cells. Paneth cells expressing EphB would detach when forced into a monolayer of absorptive progenitors expressing ephrin-B that restrict their adhesion. SCs located in + 4 positions ascending from the crypt bottom maintain stem cell features and are able to repopulate the crypt before giving rise to proliferating absorptive progenitors if Notch signalling is activated otherwise they generate tuft or enteroendocrine cells. Absorptive cell progenitors undertake a limited number of divisions before differentiating into mature enterocytes. All secretory cells are therefore generated at the crypt bottom, which is consistent with the observed decreasing number of these cells observed ascending the crypt due to the dilution effect of the proliferation of absorptive progenitors. We have used a Montecarlo model to describe the intestinal crypt as a slow dynamics system with the omission of the active forces described in individual-based models. Slow structural relaxation explains the localisation of Paneth cells to the crypt bottom without active forces and cell covering of the epithelium after cell apoptosis or anoikis. In addition, our model is scaffold free while approaches based on rigid lattices or on Voronoi tessellations are constrained to fixed shapes the size of which cannot be changed dynamically. In our model, the size of the crypt changes dynamically to respond to changes in cell production demanded by adjacent villi. By modelling this system we aim to provide novel experimentally testable hypotheses to advance in the knowledge of the mechanisms for cell proliferation, differentiation and kinetics in the crypt.

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Poster Board Number: F-1144

EX VIVO-GENERATED MOUSE MYELOID PROGENITOR CELLS MITIGATE GASTROINTESTINAL ACUTE RADIATION SYNDROME WHEN ADMINISTERED UP TO 5 DAYS AFTER IRRADIATION

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To date, there are no safe and effective drugs approved for mitigation of acute radiation injury. The aim of this study was to elucidate the potential of mouse myeloid progenitor cells (mMPC) to mitigate lethal doses of cobalt-60 gamma-radiation and kilovoltage X-rays in mouse models of acute radiation syndrome (ARS). mMPC were generated *ex vivo* from FACS sorted bone marrow hematopoietic stem cells (cKit+Thy1.1midLineage-Sca1+) from three MHC Class I mismatched mouse strains (AKRH-2k, C57Bl/6H-2b or B6.Pl-Thy1.1H-2b, and FVBH-2q). Culture-derived mMPC are restricted to the myelo-erythroid lineage and engraft across allogeneic barriers in irradiated mice to transiently give rise in vivo to granulocytes, erythroid cells, and platelets without causing symptoms of graft-versus-host disease. mMPC administration can provide the granulocytes, platelets, and erythrocytes that are lacking in pancytopenic mice upon exposure to lethal doses or irradiation. In this study cryopreserved mMPC from three donor strains with different MHC class I haplotypes were thawed and pooled for infusion into lethally irradiated unmatched CD2F1H-2d or Balb/cH-2d recipient mice across a broad range of radiation doses to assess their effect on 30-day survival. Our results demonstrate that ex-vivo generated, allogeneic mMPC significantly improve survival in both strains of mice. Survival benefit was mMPC-dose dependent and significant even when mMPC administration was delayed up to seven days post-irradiation. We further show that mMPC administration mitigates death from ARS at radiation doses of up to 15 Gy in CD2F1 mice (cobalt-60 γ-radiation); which are exposure levels that cause mortality through multi-organ failure, including major injury to the gastrointestinal (GI) tract. Even at high doses of up to 14 Gy cobalt-60 γ-radiation, mMPC administration could be delayed up to 5 days in CD2F1 mice and still provide significant benefit to 30-day survival. mMPC administration to mice 24 h after 13 Gy irradiation significantly improved integrity of the GI tract by 4 days after irradiation. This improved GI integrity in mice infused with ex-vivo generated mMPC was correlated with increased proliferation and decreased apoptosis in the jejunum four and eight days post-irradiation, a decrease in pro-inflammatory serum cytokine levels 12 days post-irradiation, and lack of bacterial translocation at any time point. These results point to a function of mMPC and their progeny outside the hematopoietic system at highly lethal radiation doses. With respect to efficacy across a broad range of radiation doses, as well as timing and practicality of administration, mMPC appear to be one of the most promising radiation countermeasure for ARS among all candidate therapeutics currently under development.

Poster Board Number: F-1145

IN VITRO CONVERSION OF GUT INTO PANCREATIC TISSUE

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Learning the rules that guide pancreas formation may eventually allow the implementation of genetic programs that induce the directed differentiation of surrogate cells towards endocrine versus exocrine gene expression programs, or induce the growth of the entire pancreatic tissue, potentially leading to cell-based therapies for diabetes. Using the genetic lineage tracing in mice, we previously showed that bHLH transcription factor ptf1a functions as a transcriptional switch towards pancreatic fate in the early pancreatogenesis. We also demonstrated that inactivation of Notch signaling causes misexpression of Ptf1a in discrete regions within the primitive stomach, duodenum and common bile duct resulting in ectopic pancreatic tissue formation in hes 1 null mice. To test directly introducing ptf1a in non-pancreatic foregut epithelium causes fate conversion to pancreas, we used adenovirus-mediated gene transfer and in vitro tissue culture. Here we show that adenovirus-mediated transient misexpression of Ptf1a in Pdx1-expressing non-pancreatic epithelium induces the trans-commitment to pancreatic fate, leading to the activation of an endogenous gene expression program in pancreatogenesis. Insulin-producing cells formed by Ptf1a transduction release hormone in response to elevated glucose, and transplantation of these cells significantly ameliorate the morbidity and hyperglycemia in mice caused by the β cell selective toxin streptozotocin. Our results suggest that the easily accessible gut epithelium may be a useful substrate for *de novo* generation of insulin-producing tissue for diabetes therapy.

Poster Board Number: F-1146

GRAFT VERSUS HOST DISEASE DISRUPTS INTESTINAL MICROBIAL ECOLOGY BY INHIBITING PANETH CELL PRODUCTION OF DEFENSINS

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Allogeneic hematopoietic stem cell transplantation (SCT) is a curative therapy for hematologic malignant tumors, bone marrow failure, and congenital metabolic disorders. However, its application is limited by the toxicity of the devastating complication, graft-versus-host disease (GVHD). GVHD is a complex process involving donor T-cell responses to host antigens and the dysregulation of inflammatory cytokine cascades. Intestinal stem cells (ISCs) play a pivotal role not only in physiologic tissue renewal but also in regeneration of the intestinal epithelium after injury. We have previously demonstrated that pretransplant conditioning regimen damaged ISCs; the ISCs however rapidly recovered and restored the normal architecture of the intestine. ISCs are targets of GVHD and this process of ISC recovery was markedly inhibited with the development of GVHD. Injection of Wnt agonist R-spondin1 protected against ISC damage, enhanced restoration of injured intestinal epithelium, and ameliorated GVHD not only in intestine but also skin and liver, which demonstrated that ISC damage plays a central role in amplifying systemic GVHD. GVHD related infection is another major obstacle to allogeneic SCT and their close relationship has been indicated in clinical settings. Septicemia is the most life-threatening infection after allogeneic SCT and gram-negative rods are the most dominant pathogens of septicemia. GVHD is one of the major predisposing factors for the development of septicemia. In this study,

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we have evaluated a link between GVHD and bacterial infection in mouse models. The intestinal microbial communities are actively regulated by Paneth cells through their secretion of antimicrobial peptides, α-defensins. Paneth cells are located besides ISCs within the crypts and identified as niche for ISCs. We discovered that Paneth cells are targeted by GVHD, resulting in marked reduction in the expression of α-defensins, which selectively kill non-commensals, while preserving commensals. Molecular profiling of intestinal microbial communities showed loss of physiological diversity among the microflora and the overwhelming expansion of otherwise rare bacteria, which caused septicemia. These changes occurred only in mice with GVHD, independently upon conditioning-induced intestinal injury, and there was a significant correlation between alteration in the intestinal microbiota and GVHD severity, indicating that these were the effects of GVHD. These results reveal the novel mechanism responsible for shift in the gut flora from commensals towards the widespread prevalence of pathogens and the previously unrecognized association between GVHD and infection following allogeneic SCT. Paneth cells are derived from ISCs and serve as niche for ISCs. Our previous and current studies addressed intestinal GVHD at the cellular level and demonstrated that ISCs and their niche, Paneth cells could survive pretransplant conditioning and regenerate injured epithelium by conditioning in the absence of GVHD. However, both ISCs and Paneth cells are targeted by GVHD, resulting in an impairment of the physiological repair mechanisms of injured epithelium. This phenomenon may explain the prolonged and refractory nature of clinical intestinal GVHD. These new insights will help to establish new therapeutic strategies that can be used to prevent and treat GVHD and related infections and improve the clinical outcome of allogeneic SCT.

Poster Board Number: F-1147

INTESTINAL SUBEPITHELIAL MYOFIBROBLASTS ENHANCES THE GROWTH OF LGR5 STEM CELLS

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Introduction: Intestinal epithelial stem cells have been an area of intense study for applications in tissue engineering and understanding intestinal diseases. Feeder support cells are used in a variety of stem cell co-cultures to sustain their growth. Intestinal subepithelial myofibroblasts (ISEMF) are the natural support cells in the *in vivo* environment. We hypothesize that ISEMF will enhance the growth of epithelial stem cells when grown in co-culture. Methods: ISEMFs and intestinal crypts were obtained using established methods from wild type and GFP C57BL/6 mice, respectively. GFP expressing crypts were mixed in Matrigel and co-cultured with ISEMF under several configurations: 1) without ISEMF as a control 2) on top of an ISEMF monolayer 3) mixed with ISEMF in Matrigel 4) above a monolayer of ISEMF but separated by a permeable membrane 5) on a permeable membrane without ISEMF as a control. Cultures were terminated after seven days and characterized through histology and qPCR assays. The growth of enteroids was quantified with qPCR of GFP DNA and through counting and size measurements. Similarly, single epithelial cells were isolated from Lgr5-GFP mice with established methods and sorted for Lgr5 and CD44 and grown on top of an ISEMF monolayer. Crypt and single stem cell co-cultures with ISEMF were also placed on PGA felt scaffolds and implanted subcutaneously in mice to assess their *in vivo* viability. Moreover, ISEMFs were transduced with lentiviruses to induce the cells to produce Wnt3a and Rspo1. Crypts were grown on top of the transduced ISEMF but without Rspo1 added to the medium. Results: Crypts grown on top of or mixed with ISEMF formed enteroids three times larger and contained six times as much GFP DNA than without ISEMF. Separating the co-culture with a permeable membrane and 1 mm distance resulted in enteroids only 1.5 times as large and with 1.5 times as much GFP DNA compared to without ISEMF. All cultures showed high expression of Lgr5 mRNA at around 10 times that of normal whole small bowel, but relatively low expression levels for differentiated epithelial markers. *In vivo* implants from both crypt and single stem cell co-cultures showed epithelial cysts with mature brush borders and goblet cells. Crypts cultured on transduced ISEMF grew to be 8 times larger than those on wild type ISEMF when they were cultured without added Rspo1 in the medium. Conclusions: ISEMF enhanced the growth of the epithelial stem cells in vitro by enlarging the size of the enteroids and supported the formation of intestinal epithelial structures *in vivo*. Rspo1 and Wnt3a transduced ISEMF supported the growth of intestinal crypts even in the absence of soluble Rspo1. This co-culture system represents a significant step toward developing the intestinal stem cell niche.

Poster Board Number: F-1148

DISREGULATED EXPRESSION OF HOX GENE FAMILY IN INDUCED PLURIPOTENT STEM CELLS FROM HUMAN NORAML AND GASTRIC CANCER CELLS KMU-CSN AND KMU-CS12

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We have generated and characterized the induced pluripotent stem cells (iPSCs) from human gastric cell line, KMU-CS12 (CS12) and from an immortal cell line, KMU-CSN (CSN), which were derived from putative human gastric stem cell/progenitor cell clone, KMU-GI2. CS12 expressed cancer cell phenotypes, i.e. the ability of anchorage-independent growth high frequency (44%) and to the expression of Oct4, a stemness marker and many types of cancer cells, and tumor development in immune deficient mice. SKY analysis indicated a characteristic duplication of the short arm of chromosome 7 to chromosome 12. Agilent Human 1A oligo-array analysis and qPCR revealed that homeobox genes like Hoxa9 (57.83 fold), Hoxa7 (32.01 fold), Hoxa4 (24.14 fold), Hoxa5 (7.24 fold) and Hoxa13 (6.14 fold) were highly expressed in CS12 cells. We also generated induced pluripotent stem cells (iPSCs) by electroporation using AP1 transcription factor Jun Dimerization protein 2 (JDP2) and Oct4. JDP2 plays roles in cell cycle regulation, cellular senescence, nuclear reprogramming and oncogenesis through the epigenetic control involved in cascades of $p19^{Arf}$-Mdm2-p53-p21-cyclin/CDK or $p16^{Ink4a}$-cyclin/CDK-RB-E2F. We found that JDP2 functioned through Wnt signal and reprogrammed CS12 and CSN to iPSCs with Oct4. The enhanced expression of Sox2 is critical for generation of CSN-iPSCs. Both iPSCs expressed three standard stemness genes like Oct4, Sox2 and Nanog, but parental CS12 and CSN did not show the alkaline phosphatase activity. Moreover, we found that original CS12 induced the tumor formation but CSN iPSCs and CS12iPSCs did not induce the tumor progression in nude mice. These results indicate that iPSCs function as possible tumor

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repressor. We also found the cross talk of Wnt signal and LIF/JAK-STAT3-Oct4 will be critical in generating iPSCs and the role of JDP2 in nuclear reprogramming and function as the tumor suppressor. We also detected the significant reduction of Hoxa 4, 5, 7 and 9 but enhanced expression of Hoxa13. Thus, the different regulation of Hoxa gene family was detected. We discuss the role of HOXA genes in nuclear reprogramming and cancer development in gastric cell line.

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NOVEL CELL SURFACE GENES EXPRESSED IN THE STOMACH PRIMORDIUM DURING GASTROINTESTINAL MORPHOGENESIS OF MOUSE EMBRYOS

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Stem cells and progenitors contribute to tissue-specific cell lineage and its homeostasis from embryonic to adult stage. These stem cells and progenitors are useful not only the understanding of organogenesis in development but also for the tissue regeneration in adulthood. Because of the lack of stem cell and progenitor markers, gastrointestinal development has not been fully understood. Especially, stomach-specific stem cell and progenitor markers have not been identified yet. In this study, we surveyed cell surface markers for the prospective stomach region during gastrointestinal morphogenesis of mouse embryo. The endoderm forms tube-like structure that spans the embryo from anterior to posterior. In E9.5 mouse embryos, the initiation of morphogenesis of gastrointestinal organs starts with the budding outward from the primitive gut, such as esophagus, stomach, and intestine, and gains defined organ-specific shape between E9.5 and E11.5. Thus, we compared these primordial gene expressions and analyzed specific cell surface markers. Using microarray-based screening, we identified 3 cell surface genes -Adra2a, Fzd5, and Trpv6- that are expressed in the developing stomach region. Interestingly, these markers can be used to specify the endoderm and mesenchyme layer of developing stomach. Adra2a, a family member of G protein coupled adrenergic receptor, was expressed in stomach mesenchyme at early development, but not late stage. Fzd5, a receptor for Wnt signaling, was expressed in pyloric endoderm and the expression was attenuated toward forestomach. Trpv6, a membrane chanel for calcium absorption, was also expressed in pyloric endoderm, but its expression continued to corpus region. Both Fzd5 and Trpv6 expression sustained until at late stage. Data from this study and others suggested that Fzd5 could function as a receptor for Wnt signaling that regulates stomach morphogenesis during development. However, the possible function of the Adra2a and Trpv6 genes during gastrointestinal tract development remain unknown. These novel marker genes will be useful in expanding our understanding of the mechanisms of gastrointestinal development. In addition, immunohistochemistry- or FACS-based analyses with antibodies against these tissue-specific cell surface markers could be useful for molecular dissection of gastrointestinal development and the analysis of stomach specific stem cells and progenitors.

Poster Board Number: F-1150

SISTER CHROMATID COHESION FACILITATES LOSS OF HETEROZYGOSITY (LOH) OF THE WILD TYPE APC ALLELE AND ITS DEFICIENCY REDUCES ADENOMA FORMATION IN APCMIN/+ MICE

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Sister chromatid cohesion is central process during DNA replication at S phase that persists until chromosome separation at the metaphase-anaphase transition. Sister chromatid cohesion is also involved in gene transcription through cohesin-dependent chromosome looping and in DNA repair by homologous recombination (HR). HR is thought to be the principle pathway to loss of heterozygosity (LOH) of Adenomatous Polyposis Coli (Apc) WT allele in adenoma formation in ApcMin/+ mice. Here, we focus on Rad21, a critical subunit of the cohesin complex, to investigate the role of sister chromatid cohesion in colorectal carcinogenesis. We propose that at the early stages of transformation sister chromatid cohesion holds two homologs together allowing HR to facilitate LOH of the wild type tumor suppressor gene Apc in colon progenitor cells. We also propose that Rad21 is an Apc-regulated Wnt signaling pathway target gene that is elevated in adenomas to further promote additional LOH and induce defective mitotic checkpoints. Moreover, sister chromatid cohesion through chromatin looping brings distant enhancers into close proximity to promoter regions to up-regulate oncogene transcription, such as c-myc gene. Results show that ApcMin/+ MEFs on a Rad21+/- background restored chromosome stability compared to ApcMin/+ MEFs alone. In our study, we also confirmed that LOH occurs in ApcMin/+ mice adenomas and in ApcMin/+ Rad21+/- mice as well. ApcMin/+ mice on a Rad21+/- background show significantly improved survival with decreased incidence of adenomas without influencing adenoma location along the small intestine and colon. Finally, AOM-induced carcinogenesis experiments suggest that ApcMin/+ mice are also protected from adenoma formation when on a Rad21+/- background and that Rad21 mRNA levels track with Wnt signaling pathway genes (Tcf4, c-myc, lgr5).

Muscle Cells

Poster Board Number: F-1151

GENERATION FUNCTIONAL MYOCYTES FROM HUMAN INDUCED PLURIPOTENT STEM CELLS

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The establishment of human induced pluripotent stem cells (hiPSCs) has made possible the patient-specific *in vitro* cell models for human disease. It is important that recreating the pathology of human disease is dependent on efficient differentiation protocols that generate relevant adult cell types from hiPSCs. Here we report the robust and reproducible differentiation of hiPSCs into mature myocytes. We demonstrate that inducible expression of MyoD in immature hiPSCs at least for 5 days could efficiently drive them into myogenic cell fate with efficiencies of 80%-90%. Induced myocytes retained their identity even after withdrawal of transgene expression, expressed mature markers for myocytes and had mature functional properties such as fusion each other and twitching by electric induction. When transplanted into immunodeficient mice, the induced myocytes could fuse with host myofiber and produced

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human Dystrophin. These findings promote modeling human muscular disease using patient-derived hiPSCs.

Poster Board Number: F-1152

HIGH EFFICIENCY DERIVATION OF SKELETAL MUSCLE PROGENITOR/STEM CELLS FROM HUMAN PLURIPOTENT STEM CELLS USING SPHERICAL CULTURES

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Human pluripotent stem cells (PSCs), such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), provide a valuable source from which skeletal muscle progenitor/stem cells (SMPCs) can be isolated. Embryoid body (EB) formation and genetic modification have been used for SMPC differentiation; however, their low efficiency is a big challenge for therapeutic applications in neuromuscular diseases. Here, we show a novel and highly efficient protocol to establish SMPCs from human PSCs using spherical cultures. For this study, we used human ESCs (WA09, H9 line) and iPSCs, which were generated from primary wild type fibroblasts by infection with lentiviral constructs encoding pluripotent genes OCT4, SOX2, NANOG and LIN28. ESC and iPSC colonies were placed directly into a culture medium containing a high concentration (100 ng/ml, usually used 20 ng/ml in standard cell culture protocols) of basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF). The colonies formed spherical aggregates termed EZ spheres. These spheres spontaneously expressed skeletal muscle progenitor markers such as Pax3 and Pax7 and could be expanded to a large scale over 10 passages by mechanical chopping using an automated tissue chopper. To determine whether the passaged progenitor cells in EZ spheres are differentiated into SMPCs, EZ spheres were dissociated, plated on coverslips, and differentiated for up to 5 weeks. The cells were fixed and immunostained using specific antibodies against muscle progenitor and myoblast markers (Pax7, MyoD, Myogenin, and Myosin Heavy Chain). EZ spheres demonstrated a high potential to differentiate into SMPCs and MyoD-positive myoblasts. Furthermore, the efficiency with which EZ spheres differentiated into muscle was significantly higher than that of EB formation in the other protocol. Furthermore, these SMPCs could form well-differentiated, multinucleated myotubes (>20% of fusion index) following 5 weeks of differentiation. Finally, we asked whether SMPCs can survive and integrate in rat skeletal muscle following cell transplantation. We injected cardiotoxin in the hind limb muscles of rats to induce focal muscle injury. After 24 hours, EZ sphere-derived SMPCs were injected into the same muscles. Immunohistochemistry using human dystrophin antibodies revealed that SMPCs could successfully integrate in the regenerating muscles at 3 weeks after transplantation. Cell survival and integration were also confirmed in the SMPC-injected muscles by using a non-invasive *in vivo* imaging system. Taken together, our new culture protocol provides potential applications of human SMPCs to treat neuromuscular diseases such as muscular dystrophy. This work was supported by grants from the ALS Association and the University of Wisconsin Foundation.

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EMBRYOLOGICAL ORIGINS OF VASCULAR SMOOTH MUSCLE CELLS MAY INFLUENCE THE DEVELOPMENT OF ATHEROSCLEROSIS

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Diseases such as atherosclerosis and aneurysms have a predisposition for specific vascular regions. Interestingly, smooth muscle cells (SMCs) from different vascular regions originate from a variety of embryological tissues. We have differentiated human pluripotent stem cells into origin-specific SMC subtypes via three intermediate populations namely neuroectoderm, lateral plate mesoderm and paraxial mesoderm. The current study aims to decipher how the different developmental pathways could contribute to varying susceptibilities of SMCs to vascular diseases. Microarray analysis of the SMC subtypes revealed distinct transcriptional signatures which retain the genetic imprints of their respective origins. Functional annotation demonstrated that lateral plate mesoderm-derived SMCs were highly enriched in genes promoting cell migration and these cells indeed displayed the greatest extent of migration in a scratch assay as compared to other subtypes. Alternatively, neuroectoderm-derived SMCs expressed genes which positively regulate cell proliferation, and cell cycle analysis confirmed that these SMCs had the greatest proportion of cells in the S phase (DNA synthesis) and G2 phase (mitosis). SMC proliferation and migration have been implicated in the development and progression of atherosclerosis. Our findings may provide insight into different mechansisms that promote disease in distinct vascular territories and explain why some vascular regions are relatively disease resistant.

Poster Board Number: F-1154

IN VITRO SCREENING FOR TRANSCRIPTION FACTORS INTERACTING WITH MYOCARDIN: NEW INSIGHTS INTO REGULATION OF SMOOTH MUSCLE CELL DIFFERENTIATION

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The proliferation and differentiation of smooth muscle cells (SMCs) play critical roles in vascular diseases. Since myocardin is a key factor in smooth muscle differentiation, in this experiment we were looking for transcription factors (TFs) or co-activators in smooth muscle differentiation signaling pathway by screening the proteins that could bind and interact with myocardin. By studying the function of the protein candidates, we may discover new molecular mechanisms of smooth muscle differentiation. After screening of 1,070 transcription factors by luciferase assay and real-time PCR, we discovered that a list of 11 TF candidates, such as zbtb43, rxra, rai14, maged1, foxo3a, could increase luciferase activity by interacting with myocardin and also could be highly expressed in mouse aorta and human SMCs in a mRNA level. We successfully constructed the Myc-Tag expression plasmids of some of the TF candidates and further confirmed by co-immunoprecipitated assay (co-IP) that myocardin could bind with 4 TFs (RAI14, MAGED1, ZBTB43 and FOXO3A). Moreover, the expression level of above 4 TFs was significantly upregulated during the process of mouse embryonic stem cells differentiating into SMCs induced by 10^{-5}M all trans-retinoic acid. All these data suggested that these newly discovered TFs

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might play an important role in the SMC differentiation signaling pathway though interacting with myocardin.

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EXTENDED LIFESPAN AND DELAY OF SYMPTOMS BY NEUROTROPHIC FACTORS COCKTAIL SECRETED FROM MOUSE MUSCLE PROGENITOR CELLS IN ALS MICE

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Background: Neurotrophic factors (NTFs) preserved and protected motor neuron in ALS models. However, all the clinical studies with administration NTFs in ALS patients failed. We have developed muscle progenitor cells (MPCs) populations expressing BDNF, GDNF, VEGF or IGF-1, (MPC-NTFs). Combined conditioned media collected from the cells rescued motor neurons cell line (NSC-34) from various insults. Furthermore, MPC-NTFs transplantation enhanced the regeneration of rat sciatic nerves after injury. Here we examined the retrograde transport of NTFs along motor neuron's axons and tested whether MPC-NTFs transplanted into muscles can improve the symptoms and survival of SOD1 mice. Results: A mixture of MPC populations each expression one of the of the four NTFs was transplanted into the hind legs of SOD1 mice on days 90,104, 118 of life. We found a significant delay of the symptoms (up to 30 days in the male) and extension of lifespan (12 days in the males and 18 days in females). However, transplantation of MPC alone or MPC overexpressing just GDNF didn't elicit any improvement. The results suggest a synergistic effect of the transplantation of MPCs expression several NTFs. In order to study the possible synergistic effect of the mixture of MPC-NTFs on a signal transduction pathway; we focused on the PI3K- AKT motor neuron survival pathway. We found that the supernatant of a mixture of condition media from MPC populations expressing NTFs increase the phosphorilated AKT by 6-8 folds compare to MPC expressing a single NTF. In order to study the possible axonal transport to the cell body we conjugated Qdot605 streptavidin to biotinylated GDNF and added it to axon endings of primary motor neurons cultured in compartmental microfluidic devices. Strikingly we observed that the Qdot-GDNF undergo highly directed axonal transport in the retrograde direction and to accumulate in the cell body compartment. Discussion and conclusions: Here we have build a novel powerful strategy enable a stable, long-term administration of four NTFs factors cocktail. Since intramuscular inoculated muscle progenitor cells participate in the formation of post mitotic multinucleated fibers, this route of administration of genetically manipulated MPCs results in a stable, long-term expression of the four NTFs. The constant and continuous releases of the critical NTFs from the muscle fibers through the neuromuscular junction into the motor neuron system, and the retrograde transport to the cell bodies in spinal cords probably inhibit death pathways. We hope that our study will lead to a novel strategy to slow the progress and alleviate the symptoms of ALS, and extend the life expectancy and quality of affected patients.

Poster Board Number: F-1156

HETEROGENEITY AMONG ADULT MOUSE SKELETAL MUSCLE STEM CELLS: A SUBPOPULATION RETAINS ALL TEMPLATE DNA STRANDS AFTER CELL DIVISION

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Satellite cells are adult skeletal muscle stem cells that are quiescent and constitute a poorly defined heterogeneous population. Upon muscle injury, satellite cells enter the cell cycle, generate myoblasts that will effect tissue repair by myofibre production, and then self-renew. Satellite cell ablation experiments have shown that this stem cell population is critical for regenerative myogenesis. Using transgenic Tg:Pax7-nGFP mice we show that Pax7-nGFPHi quiescent satellite cells are less primed for commitment, have a lower metabolic status, and have a delayed first mitosis compared to Pax7-nGFPLo cells. Serial transplantations of these subpopulations in mice show that Pax7-nGFPHi can give rise to Pax7-nGFPLo cells, and each can serially engraft for up to 6 passages. After satellite cell activation, proliferating Pax7-nGFPHi cells continue to exhibit lower metabolic activity. Notably, the majority of the cells in this subpopulation perform asymmetric DNA segregation during cell division. Interestingly, daughter cells retaining template DNA strands express stem cell markers whereas daughter cells inheriting nascent DNA strands express differentiation markers immediately after cell division. Using chromosome orientation-fluorescent *in situ* hybridization, we demonstate for the first time that all chromatids segregate asymmetrically in Pax7-nGFPHi cells, whereas Pax7-nGFPLo cells perform random DNA segregation. We propose that during homeostasis, quiescent Pax7-nGFPHi cells represent a novel reversible dormant stem cell state distinct from the remaining quiescent muscle stem cells. Furthermore, after acute trauma Pax7-nGFPHi cells generate distinct daughter cell fates during muscle regeneration and asymmetrically segregate template DNA strands to the stem cell. These findings provide major insights into the biology of stem cells, and those that segregate DNA asymmetrically.

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ENGRAFTMENT OF MOUSE FETAL MYOGENIC PROGENITORS INTO DYSTROPHIC MUSCLES

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[Purpose] Duchenne muscular dystrophy (DMD) is caused by the mutations of the gene encoding dystrophin. Although myoblasts isolated from adult skeletal muscle tissues have been used to treat DMD, these cells exhibit poor capacity for the engraftment into regenerating muscle because the transplanted cells loss the contribution to muscle stem cell. Exploring myogenic progenitor/stem cells suitable for cell therapy to DMD, we examined capability of Pax3-positive embryonic, fetal, and adult skeletal muscle progenitor/stem cells for the engraftment to skeletal muscle of DMD-model mice. [Materials and Methods] Pax3GFP/+ mice were produced by the targeted insertion of the gene encoding green

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fluorescent protein (GFP) into the Pax3 locus. Embryonic skeletal myogenic progenitors (EMPs) were isolated manually from somites of Pax3GFP/+ embryos at E10.5. Fetal skeletal myogenic progenitors (FMPs) were isolated from limbs and diaphragms of Pax3GFP/+ fetuses at E16.5. Adult muscle stem cells, satellite cells (SCs), were isolated from diaphragms and abdominal muscles of 4-week old Pax3GFP/+ mice as previously reported. [Results] To obtain populations of EMPs, FMPs, and SCs, (Pax3)GFP-expressing cells were purified by fluorescence-activated cell sorting from Pax3GFP/+ mice respectively. To evaluate the therapeutic potential of EMPs, FMPs, and SCs in animal models of muscular dystrophy, these cells were injected into tibialis anterior (TA) muscles of DMD-null host mice, which completely lack dystrophin and are used to DMD-model mice. Intra-muscular transplantation of FMPs and SCs into cardiotoxin-injected TA muscles of dystrophic mice resulted in efficient engraftment of myofibers with restoration of dystrophin, whereas no dystrophin positive myofibers have been observed in EMPs-transplanted TA. To compare the myogenic potentials in vitro among EMPs, FMPs and SCs, these cells were grown for 7 days with skeletal muscle proliferation and differentiation medium, respectively. EMPs failed to grow under this culture condition. As previous report, SCs displayed a myogenic phenotype in culture, expressing myogenic regulator factors including MyoD and Myogenin, and differentiating into multinuclear myotubes expressing Troponin T, which is one of differentiated skeletal muscle markers. FMPs also generated multinucleated myotubes expressing Troponin T. Almost all (>98%) FMPs expressed MyoD after 2 days, and Myogenin after 5 days. Taken these date together, FMPs had myogenic potential *in vivo* and in vitro as well as SCs had. [Conclusion] The data here demonstrates that (Pax3)GFP positive FMPs have a therapeutic potential to restore dystrophin expression in muscular dystrophy. Understanding the cellular and molecular mechanisms underlying efficient transplantation of FMPs and SCs would help development of cell therapies for skeletal muscle degeneration diseases.

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ROLE OF PANNEXIN 1 IN SATELLITE CELL ACTIVATION AND DIFFERENTIATION

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Adult skeletal muscle conformed by large post-mitotic multinucleated fibers have the ability to regenerate and recover from injury by activating a small population of muscle progenitor cells named satellite cells. Upon activation, satellite cells have the ability to self-renew and differentiate into new muscle fibers. Satellite cell activation and differentiation is a highly coordinated process that requires cytoskeleton rearrangements and cell-cell communication. Pannexins are novel family of hemichannels proteins that are associated with cell-cell communication and ATP exchange. Previous research shows that Pannexin1 is expressed predominantly in brain and muscle; however, its role in muscle remains undefined. In this study, Panx1 expression was monitored in mouse primary myoblast to investigate Panx1 role during the activation and differentiation processes. These cells were maintained in an undifferentiated, proliferating stage in growth medium and induce to differentiate by replacement of fetal calf serum with adult horse serum. Western blot analysis and immunostaining revealed that undifferentiated, proliferating primary myoblasts have no or very little Panx1 expression. In contrast, activated myoblasts show robust Panx1 expression as early as 3 hours after induction and its expression persisted in differentiated myoblasts. The particular timing of Panx1 appearance in the cells correlates with time of cytoskeleton rearrangements and initiation of satellite cell migration, suggesting that Panx1 has an important role in satellite cell differentiation and possibly migration. Currently, we are examining primary myoblasts transfected with siRNA to investigate Panx1 downregulation during the differentiation process.

Endothelial Cells/Hemangioblasts

Poster Board Number: F-1161

HUMAN PLACENTAL ENDOTHELIAL PROGENITOR CELLS ACCELERATE SKIN WOUND ANGIOGENESIS AND HAVE SYNERGISTIC ACTION WITH MESENCHYMAL STEM CELLS.

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Background: Cellular therapy of cutaneous wounds is an established method with commercially available products. Although these therapies include often epidermal stem cells or progenitors or dermal fibroblast or mesenchymal stem cells, they often fail to directly address one of the key issues in many chronic wounds which is the lack of angiogenesis. Angiogenesis has been attributed to a progenitor known as endothelial progenitor cells (EPCs). Due to the large vascular capacity of the placenta, we propose that a rich population of EPCs would be present and could be isolated for use in a therapeutic setting for wound healing. Methods: EPCs were isolated from human term placentas (gestation 37-38 weeks), obtained from the Royal Brisbane and Women's Hospital following caesarean section. Enrichment of the EPCs population and elimination of potential contamination with haematopoietic and mesenchymal stem cells (MSCs) was conducted by positive selection of CD34+ and depletion of CD45+ cells via MACS purification. nu/nu mice were injected subcutaneously, eight hours after wound formation, with either single populations of EPCs, MSCs or as a dual combination of EPC/MSCs. Assessment of wound healing was conducted on day 7 post injection. Results: Local administration of single EPCs and MSCs doses showed no significant increase in wound acceleration in comparison to controls. However, EPC administration lead to an increase in murine CD31 surface marker, a measure for new vessel formation, demonstrating a likely paracrine influence of EPCs in wound healing. The combination of EPC/MSCs accelerated wound healing, demonstrating possible synergistic effects to wound healing. Conclusion: The human term placenta is an accessible and abundant supply of EPCs, which have been shown to have promising effects in improving wound healing either as a single dose or as a co-therapy with MSCs.

Poster Board Number: F-1162

MECHANISMS INVOLVED IN THE EFFICIENT DIFFERENTIATION OF HUMAN FETAL ENDOTHELIAL CELL-DERIVED INDUCED PLURIPOTENT STEM CELLS (IPSCS) TOWARD ENDOTHEDIAL CELLS

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Reprogramming of somatic cells using a few transcription factors has been shown to be a robust and efficient method of producing pluripotent stem cells. Currently, a large number of studies on these induced pluripotent stem cells (iPSCs) are increasingly

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focused on clinical applications of these cells. As with human embryonic stem cells (hESCs)_the 'gold standard' for pluripotent stem cells_iPSCs have the ability to differentiate towards lineages of all three germ layers and germ cells, making these cells valuable for generating specific, functional cells for therapeutic application. One of the most clinically relevant somatic lineages is endothelial cells (ECs), which can be used therapeutically to treat ischemic diseases of many organs including ischemic heart disease, stroke, and peripheral vascular disease. hESCs are clearly able to generate this lineage; however, a technically demanding, multistep process is required and the final yield is low. We have previously reported that human umbilical venous endothelial cells (HUVEC) can be used to generate iPSCs with the use of only two factors, OCT4 and SOX2. These 2 factor-HUVEC derived iPSCs (2F-iHUV) are able to undergo tri-germ layer differentiation both in vitro and *in vivo* in terms of teratoma formation. Since there is some recent data to suggest that iPSCs can retain epigenetic memory of the somatic cell from which it was generated from, we were curious to whether 2F-iHUV would be more efficient at differentiating into ECs compared to hESCs. We found that 2F-iHUV can acquire an EC phenotype without the need for embryoid body formation and collagen IV, in contrast to hESC derivatives, and within a very short duration (<1 week). Surface marker detection revealed that when cultured in EC medium, 2F-iHUV derivatives rapidly become positive for CD31/PECAM-1 and CD144/VE-cadherin, two EC markers. These differentiated 2F-iHUV EC-like cells additionally express other endothelial markers including von Willebrand Factor (vWF) and the VEGF receptor FLK1. Furthermore, 2F-iHUV EC-like cells can form tubal structures when plated on Matrigel and uptake acetylated-low density lipoproteins, both EC functional assays. To explore the specific mechanisms involved in the efficient EC differentiation of 2F-iHUV, we searched for the up-regulation of EC-transcription factors and found that FOXC1, FOXC2, and ER71 were expressed in these cells, which can further be increased by culturing these cells in EC medium. In summary, we found that HUVEC is a good source for iPSC generation and the iPSCs generated from HUVEC can differentiate to an EC lineage more efficiently. These findings suggest 2F-iHUV may be an efficient model for studying EC differentiation as well as a cell source suitable for generating EC for therapeutic application.

Poster Board Number: F-1163

A ROLE FOR SOX7 IN THE FORMATION AND MAINTENANCE OF HEMOGENIC ENDOTHELIUM

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During embryogenesis the first hematopoietic cells are derived from the hemangioblast through the formation of the intermediate stage named the hemogenic endothelium. Recent findings suggest that the transcription factor Sox7 might be implicated in the cell fate specification and/or survival, and expansion of this specialized endothelium. To address this question, we aimed to characterise cells expressing Sox7 at the onset of hematopoietic development using the *in vitro* differentiation of embryonic stem (ES) cells as a model system. Tracking and isolating Sox7 expressing cells was performed using an ES cell line carrying a bacterial artificial chromosome (BAC) transgene with the first exon of Sox7 replaced by a GFP-Neo cassette. Time-course analyses revealed that Sox7 expression was up-regulated during the transition from hemangioblast to hemogenic endothelium. Interestingly, the SOX7-GFP+ population co-expressed Tie2, VE-cadherin, c-Kit and CD31 cell surface markers - an immuno-phenotype reminiscent of the hemogenic endothelium cell population. Importantly, SOX7-GFP+ cells gave rise to all primitive and definitive hematopoietic colonies in semi-solid culture. Finally, inducible knock-down studies revealed the dramatic effect of Sox7 deficiency on the emergence of the hemogenic endothelium cell population. We have previously demonstrated the critical requirement for a transient expression of SOX7, as maintenance of its expression impaired blood cell formation. In order to define the molecular mechanisms involved in the tight regulation of Sox7 expression at the onset of hematopoiesis, we have defined the regulatory regions controlling its transcription. The first one covers the promoter region of Sox7; the second one, located outside of the coding sequence within the 3' UTR, most likely presents regulatory function. Analyses of fragments covering the Sox7 promoter regions showed a strong activity in luciferase reporter assays. Moreover, co-transfection with Gata2, Scl and Ets expressing vectors further enhanced this activity in HEK 293T cells, suggesting a positive regulation of Sox7 transcription by these factors. In summary, we demonstrated a crucial role for Sox7 in the formation and/or specification of hemogenic endothelium cell population. Better knowledge about the mechanisms regulating the formation of the hematopoietic precursors is crucial for understanding the molecular abnormalities resulting in blood disorders as well as for further development of cell replacement therapies.

Poster Board Number: F-1164

A DEVELOPMENTAL ROLE FOR CIRCULATING HUMAN ENDOTHELIAL PROGENITOR CELLS IN MID-TRIMESTER FETUSES

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Aim: Endothelial Colony Forming Cells (ECFC) represent a set of circulating progenitor cells capable of rapid expansion and generation of endothelial progeny, with purported roles in vascular homeostasis and repair in post-natal life. We hypothesise ECFC to exist in fetal circulation. Extending on the observation that endothelial cells perform key regulatory roles in tissue development, we further hypothesise a specific role for circulating ECFC in organogenesis. Methods: ECFC were isolated from umbilical cord blood and mid-gestation fetal blood by adhesion selection in endothelial growth media. To verify ECFC character, immunocytochemistry (ICC) was first employed to demonstrate the expression of endothelial markers. Subsequently, colony forming assays were conducted to assess clonogenic character, and Matrigel tube-forming assay was carried out to compare the vasculogenic potential of the cell sources. Subsequently, cells were injected into a murine model of hindlimb ischaemia to study the ability to effect rescue. Finally, genome-wide microarray analysis was carried out to compare the expression profiles of endothelial cells derived from both cell populations and identify significant gene ontologies associated with development. Results: ECFC were successfully isolated from fetal blood as assessed by ICC. However, outgrowth cells derived from fetal blood were found to be highly heterogeneous and found to express lower levels of CD31, CD34, CD133, HLA-1 and HLA-2. In addition, in the colony forming assays, 60% colonies generated in the fetal blood group were found to be greater than 5 mm in diameter, as compared to none in the UCB-EPC group. When placed in Matrigel, fEPC were found to be highly vasculogenic, and established more complex, macroscopic networks capable of secondary aniogenic sprouting. Increased functionality was also demonstrated in the ability to rescue ischaemia, with fEPC capable of restoring perfusion levels to 56% of normal within 16 days (Saline: 26.8%, UCB-EPC:

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37.8%). These results were verified by micro-CT angiography, confirming restoration of deep-tissue perfusion. Crucially, immunohistochemistry was employed to demonstrate chimeric capillary networks in the ECFC-injected groups, demonstrating the differentiation and functional integration of ECFC from both sources, and verifying the EPC character. Through microarray analysis, 2123 genes were found to differentially regulated by two-fold in fetal blood-derived endothelial cells. Gene ontology analysis reveal several of these genes to be involved in developmental organogenesis. Pathway analysis suggests the involvement of six pathways, including the NOTCH, TGFBR and Wnt pathways, key pathways in tissue and organ development. Conclusion: ECFC were successfully isolated from mid-gestation fetal circulation, which are phenotypically and functionally different from umbilical cord-blood derived counterparts. Comparative microarray analysis on endothelial cells derived from fetal blood and umbilical cord blood indicates a role for ECFC in organogenesis during fetal development.

Poster Board Number: F-1165

FUNCTION OF GFI1 AND GFI1B IN THE EARLY HEMATOPOIESIS

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The molecular and cellular mechanisms leading to the formation of blood progenitors are still poorly understood. Previous studies have established that the transition from hemangioblast to hematopoietic progenitors proceeds through a hemogenic endothelium intermediate stage. This process was named endothelial to hematopoietic transition (EHT) and is critically dependent on the activity of the transcription factor RUNX1. We have recently identified GFI1 and GFI1B transcription repressors as major players in this process. It was demonstrated that GFI1 and GFI1B are direct transcriptional targets of RUNX1 and we established furthermore by gain of function that GFI1 and GFI1B are able to trigger in the absence of Runx1 the formation of round blood progenitors. In these studies, we have investigated, by loss of function, the respective and cumulative roles of the GFI1 and GFI1B transcription factors *in vivo* during yolk sac and embryo proper hematopoiesis. We demonstrate that both genes are expressed in the same Tie2+c-Kit+CD41+ cell population during early embryogenesis. As the single knock-out (KO) of Gfi1 and Gfi1b genes do not recapitulate the Runx1-/- phenotype, we evaluated a potential compensation of GFI1 or GFI1B deficiency by each other. We therefore investigated the phenotype of Gfi1/1b double KO (Gfi1GFP/GFP/Gfi1bGFP/GFP, KO/KO) in which the Gfi1 and Gfi1b genes have been replaced by GFP. We observed that GFP+ cells in the KO/KO embryos, including CD41+ hematopoietic cells, are only present in restricted areas of the yolk sac, corresponding to the initial sites of hematopoietic development. In contrast in healthy as well as single KO counterparts these cells are disseminated within the whole yolk sac and embryo proper. Moreover, we demonstrated that blood progenitors generated in the KO/KO embryos maintain the expression of endothelial genes and are able to generate blood cells. Consistent with a compensation mechanism, we noticed, with either reporter mouse lines or quantitative PCR, that in the absence of Gfi1b, Gfi1 is significantly expressed, while its expression could not be detected in the presence of Gfi1b. The comparison of transcriptome from E8.5 KO/KO embryos to their heterozygous littermates identified changes in expression of genes implicated in cellular growth, proliferation and movement as well as hematopoietic system development. Furthermore, we recently derived embryonic stem cells from the KO/KO embryos and preliminary in vitro experiments support the requirement for the GFI1 and GFI1B transcription factors in hematopoietic development we established *in vivo*. In conclusion, our studies identify GFI1 and GFI1B as critical players in the transition between endothelial and hematopoietic cells associated with the down-regulation of the endothelial genes and dissemination of blood precursors within the developing embryos. Our findings also suggest that GFI1B is more important at early stages of development than GFI1, repressing the expression of the latter in the cells. These new insights in the mechanisms of hematopoietic development should be instrumental in future generation of blood cells for cell replacement therapies as well as in our understanding of molecular abnormalities resulting in blood diseases.

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SMALL RNA SEQUENCING IDENTIFIES MICRORNAS INVOLVED IN THE DISTINCT BIOLOGICAL FEATURES OF HUMAN ENDOTHELIAL PROGENITOR CELLS FROM DIFFERENT ANATOMIC LOCATIONS

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Endothelial progenitor cells (EPCs) play a fundamental role in post-natal vascular repair, yet EPCs from different anatomic locations possess unique biological properties. The underlying mechanisms are unclear. Here we performed transcriptome analysis for EPCs isolated from 2 different sources: cord blood (CB) or adult peripheral blood (PB). EPCs from CB express abundant genes involved in cell cycle, hypoxia signalling and blood vessel development, correlating with the phenotypes that CB-EPCs proliferate more rapidly, migrates faster, and form tubule structure more efficiently. Small RNA sequencing (smRNA-Seq) deciphered microRNAs (miRNA) patterns in EPCs isolated from CB and PB: 54 miRNAs were enriched in CB-EPCs, while another 50 in PB-EPCs. Specifically, CB-EPCs express more angiogenic miRNAs such as miR-31, while PB-EPCs possess more tumor suppressive miRNAs. Knocking down miR-31 levels in CB-EPCs suppressed cell migration and microtubule formation. Novel, previous unannotated miRNAs were also found from smRNA-Seq. RNA-IP verified the association of novel miRNAs in Ago2-positive complexes, and functional assays assigned pro- or anti-angiogenic function to EPC novel miRNAs. Our results show the foundation for a more detailed understanding of EPCs and the roles of miRNAs in post-natal angiogenesis. Stimulating the expression of angiogenic microRNAs and genes in EPCs of low activity (such as those from patients with cardiovascular diseases) might allow the development of novel therapeutic strategies.

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Poster Board Number: F-1167

COMPARATIVE STUDY OF HEMATOPOIETIC LINEAGE POTENTIAL OF IPS CELLS DERIVED FROM TWO MESODERMAL CELL LINEAGES

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Identifying the key regulatory genes that direct human ES/iPS cell differentiation to certain lineages is required before considering these cells for their potential clinical application. It has been proposed that the cellular starting material used to generate novel iPS cell lines imparts an epigenetic memory which in turn influences the iPS lines' potential to differentiate towards certain developmental lineages. To determine novel key regulators that direct ES/iPS cell differentiation to hematopoietic lineages, we decided to compare differentiation capacity of multiple iPS cell lines generated from two neonatal mesodermal tissue derived cell types. For this, we generated multiple iPS cell lines from umbilical cord blood derived endothelial cells and amniotic fluid derived mesenchymal stem cells, using an identical dedifferentiation system/protocol. Despite the similar germ layer origin of these iPS derived cell lines, these lines showed significant differences in blood generation efficiency and lineage specification capacity. From the 8 cord blood derived iPS cell lines we tested, the average efficiency of hematopoietic (CD45+) cell generation was 34.6+/-21.2% (range 9.8 to 65.8%), of which 5.8+/- 4% were CD45+CD34+ hematopoietic progenitors. From the 9 mesenchymal stem cell derived iPS lines, the average efficiency of CD45+ hematopoietic cells was 14.2+/-9% (range 1.6 to 26.3%), of which 2.9+/- 1.9% were CD45+CD34+ hematopoietic progenitors. Note, using our standardized iPS-2-Blood differentiation protocol the efficiency of blood differentiation for individual iPS cell lines was highly reproducible. We further compared the iPS cell lines for their potential to differentiate into hematopoietic cells of the myeloid, erythroid, and lymphoid lineages. Both mesoderm lineage derived iPS cell lines showed myeloid lineage differentiation potential as evidenced by CFU assay for presence of macrophages and granulocytes. However, erythroid (BFU-E) potential was severely compromised in the cord blood derived iPS cell lines compared to amniotic fluid derived iPS cell lines. Preliminary studies also suggest a block in T cell development in the cord blood derived iPS cell lines at a specific stage (CD3, CD4 double positive). Dually impaired erythropoiesis and lymphopoiesis in several diseases has previously been demonstrated to be due to a specific mitochondria dysfunction. We are currently investigating if the starting cell material used to generate the iPS cell lines can have residual effects on mitochondrial function, in the context of epigenetic memory, via gene expression, protein expression and epigenetic analyses. The comparative analysis of the iPS cell lines at both transcriptional and posttranslational level will likely provide novel insights into some of the important mechanisms in hematopoiesis. The close ontogenological relationship of the two mesoderm derived cell types should facilitate the deconvolution of the molecular basis of the differences seen in differentiation potential.

Poster Board Number: F-1168

TRACKING THE EMERGENCE OF PRIMITIVE ERYTHROCYTES IN DIFFERENTIATING MOUSE EMBRYONIC STEM CELLS AND DURING MAMMALIAN EMBRYOGENESIS

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Primitive erythrocytes are the first blood cells to emerge during mammalian embryogenesis. They appear in the yolk sac, where blood islands consisting of both hematopoietic and endothelial cells develop around day 7.5 of gestation. Close proximity of both endothelial and hematopoietic lineages within blood islands has lead to the hypothesis that they arise from a common precursor - a mesodermal cell termed 'hemangioblast'. The in vitro counterpart of the haemangioblast - the blast colony forming cell (BL-CFC) - was identified during embryonic stem (ES) cell differentiation. This progenitor cell gives rise, after 4 days of culture, to blast colonies with hematopoietic, endothelial and vascular smooth muscle potential, thus, enabling us to study the process of differentiation of multiple lineages in vitro. Recently, both *in vivo* and in vitro studies have demonstrated that the hemangioblast generates hematopoietic cells via an intermediate stage - 'hemogenic endothelium', which represents a specialised endothelial cell population with hematopoietic potential. In order to track the emergence of primitive erythrocytes we engineered by recombineering a Bacterial Artificial Chromosome (BAC) containing the eGFP gene driven by the embryonic globin βH1 promoter, specifically active in primitive erythrocytes. We then generated, with this BAC, a transgenic ES cell line, as well as the corresponding mouse line. When βH1-eGFP+ cell populations were isolated in vitro and *in vivo*, we found that they contained primitive erythroid progenitors, indicating that βH1 is expressed from the onset of commitment to the erythroid lineage. Time lapse imaging and FACS analyses indicated that cells expressing βH1-eGFP emerge around day 1.5 of blast colony development in vitro within a CD41+ cell population. Further analysis both *in vivo* and in vitro demonstrated that these primitive erythroid cells are initially generated from a progenitor cell population expressing TIE2, C-KIT and negative/positive for CD41, a phenotype of hemogenic endothelium cell population. The cell population that expresses TIE2, C-KIT, CD41 and βH1-eGFP gives rise to primitive erythroid colonies in vitro and has a potential to generate endothelial tubes in in vitro cultures. In contrast, the corresponding βH1-eGFP negative cell population generates definitive hematopoietic colonies. These results indicate that primitive erythrocytes are generated from haemogenic endothelial cells and suggest that the βH1-eGFP+ cell population represents the earliest point of restriction to primitive hematopoietic fate. Gene expression analyses between the different hemogenic endothelium cell populations were performed. Identification of genes specifically expressed by distinct populations will help us to understand the molecular factors involved in the specification of primitive and definitive hematopoietic lineages from the hemangioblast.

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Poster Board Number: F-1169

SENESCENCE OF ENDOTHELIAL PROGENITOR CELLS DURING EX-VIVO EXPANSION IS MEDIATED BY NFKB AND INFLAMMATORY CYTOKINES

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The clinical value of Endothelial Progenitor Cells (EPCs) is underscored by their capacity to promote vascular repair of ischaemic tissues. One of the difficulties of EPC therapy is generating sufficient numbers of cells; therefore *ex vivo* expansion methodologies are often used to amplify cell numbers. This study characterises EPC growth dynamics in culture conditions, ensuing senescence, and how this impacts on vasoreparative function. The term EPC describes a diverse range of different cell types. Therefore, we have focused on one EPC subtype, Outgrowth Endothelial Cells (OECs), which are widely accepted as a bona fide progenitor fully committed to the endothelial lineage. OECs were isolated from peripheral and umbilical cord blood following well-established protocols. OECs exhibited significant expansion capability *ex vivo*, although their proliferative capacity progressively declined with serial passages, eventually reaching a Hayflick limit of approximately 30 and 60 population doublings for peripheral and umbilical cord blood-derived OECs, respectively. Growth curves and FACS analysis demonstrated a permanent cell cycle arrest in G1 associated with a significant decreased of BrdU uptake ($p<0.01$) at higher passages. These OECs displayed a significant increase of cytoplasmic volume linked to the expansion of mitochondrial and lysosomal mass in unison with increased beta-galactosidase activity ($p<0.01$) and significant accumulation of gamma-H2AX foci ($p<0.01$). Furthermore, there was a significant decrease in telomerase activity coupled with telomere shortening, indicating a process of replicative senescence. Whole-genome transcriptome array identified 828 significantly over-expressed and 705 under-expressed transcripts when comparing early passage (EP) versus senescent OECs. Further bioinformatics analysis highlighted a compelling inflammatory component in senescent OECs. qRT-PCR validated microarray results by revealing a significant increase in gene expression levels of IL1A, IL1B, IL6, and IL8. Protein arrays confirmed that senescent OECs exhibited higher IL8, IL1B, and CCL2 protein levels, and concomitantly their secretome was highly enriched for IL6, IL8, CCL2, and CXCL1 when compared to EP-OECs. As NFkB plays an essential role in regulating inflammation, we tested whether blocking transcriptional activation affects OEC growth. Interestingly, when OECs were cultured in the presence of sulfasalazine (NFkB inhibitor), there was a significant increase in OEC replicative capacity. Functional assays were used to compare EP-OECs with senescent counterparts. In vitro models indicated that senescent OECs had significantly impaired capacity to migrate and form tubes compared to EP controls ($p<0.05$). OECs are known to promote re-perfusion in murine ischaemic retinopathy and this model was used to evaluate the regenerative potential of senescent OECs in vivo. Senescent OECs demonstrated impaired capacity to integrate and re-vascularise ischaemic retina compared to EP-OECs. In conclusion, EPCs have limited replicative potential and become senescent after long term culture and these cells display impaired vasoreparative function in vitro and in vivo. The senescent phenotype is associated with the elaboration of pro-inflammatory cytokines under transcriptional control by NFkB. Therefore cell passage and a senescence phenotype are important factors to consider when harnessing EPCs for cytotherapy.

Poster Board Number: F-1170

IMPACT OF VASCULAR ENDOTHELIAL GROWTH FACTOR BOUND STENT ON CAPTURING ENDOTHELIAL PROGENITOR CELLS : EVIDENCE IN PORCINE CORONARY MODEL

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Background: Recently developed EPC capturing stent, on which surface is coated with anti CD34 antibody, appears to accelerate tissue formation but does not reduce intimal hyperplasia. Since CD34-positive cells to be captured contain various cell types such as smooth muscle progenitor cells beside EPCs, degree of selective capturing of EPCs must be not so high. On the other hand, VEGF receptor is exclusively expressed on endothelial lineage cells. In this study, VEGF-bound stent was newly devised aiming at highly selective capturing of EPCs, and *in vivo* performances in early period of stenting were evaluated in porcein coronary model in comparison with those of anti CD34 antibody-bound stents. Methods and Results: Metallic stent (diameter 2.5-3.5mm, length 8mm) were coated with vinyl alcohol copolymer, followed by activation of surface hydroxyl group and subsequent protein binding (VEGF-bound stents n=39 and anti porcein CD34 antibody-bound stents n=33) were in-house prepared. At 2, 14 and 42 days after stenting, coronary arteries were harvested. At 2 day-stenting of VEGF-bound stent, a low population of KDR -positive cells adhered on stent struts were observed. At two weeks, stent struts were covered by newly regenerated tissues which were immune-fluorescently positive for CD34 and KDR. At 2-week, scanning electron microscopic images showed that uniform monolayerd tissue formed on struts is morphometrically resembled to native endothelium and exhibited continuous tissue appearance with native tissues. On the other hand, for anti CD34 antibody-bound stents, high population of adhered cells at 2-day stenting and non-uniform multilayered cell aggregates at 2 weeks stenting were observed, although endothelial-like cell-based tissue was also formed here and there. Neointimal thicknesses at 2-week and 6-week stenting appeared to be low for both stents (67.9±27.3μm vs 79.8±25.2μm at 2 week, 203±77.2μm vs 218±26.9μm at 6 week). Conclusion: VEGF-bound stent provides highly selective capturing of EPC followed by accelerated endothelial tissue formation compared with anti CD34 antibody-bound stents. Rapid, high quality and intact endothelium tissue formation provides beneficial therapeutic option for cardiovascular stenting.

Poster Board Number: F-1171

IN VIVO HIERARCHY OF RESIDENT ENDOTHELIAL PROGENITOR CELLS INTEGRAL TO SKIN WOUND ANGIOGENESIS.

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Background: Although substantial progress has been made to further understanding the biology of endothelial progenitor cells (EPC), this field remains controversial. This arises from the lack of clarity in defining EPC. Often cells that promote angiogenesis, like myeloid and mesenchymal stem cells, have been labeled as EPC. Although recently developed culture assays have confirmed a proliferation and differentiation hierarchy of endothelial cells in vitro, it is not known whether this hierarchy exists *in vivo* at sites of

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active angiogenesis. Moreover, the origin of the EPC participating in endogenous tissue vascularization is debated. Our initial objective was to establish a hierarchy of endothelial cells *in vivo* during wound-associated angiogenesis. A second aim was to determine whether these cells were originating from a distant site, such as the bone marrow, or whether they were locally derived. Methodology: To track EPC *in vivo*, wounds, blood and bone marrow were collected from D1-7 and tissues were applied to a multi-parameter flow cytometry to exclude CD45+ cells and simultaneously track 3 markers often associated with EPC; CD34, VEGFR2 and CD31. Results: 3 distinct endothelial populations were identified in healing wounds according to different combinations of our markers. All were CD45lo or negative. Kinetics of these populations suggested a model of progenitor, transit amplifying and differentiated type of distribution. The progenitor (P) population (CD45loCD34+CD31negVEGFR2neg) remained constant in number during healing, suggesting self-renewal, but also gave rise to 2 distinct populations that expanded from D2 to D5; a transit-amplifying (TA) population, CD45loCD34+CD31loVEGFR2lo, and a terminally-differentiated (D) population, D45loCD34+CD31hiVEGFR2hi. This hierarchy of endothelial maturation was confirmed via, cell cycle analyses, immunohistochemistry and mathematical modelling. Applying this technique to a model of wound healing with defective angiogenesis we showed that diabetic ob/ob mice were EPC replete. However, the TA population was absent and the D population had markedly reduced VEGFR2 levels (p=0.01), implicating inhibition of endothelial maturation in their vascular defects and not an absence of progenitors. Study of GFP+ bone marrow chimeras demonstrated that these endothelial populations were locally derived. This was also confirmed by examining the kinetics of these 3 populations in the bone marrow and blood during wound healing. Conclusion: Our results identify a new hierarchy of EPC to mature endothelial cells *in vivo*. The maturation of EPC is absent in a pathological setting, and suggest a prominent role for skin resident EPC in wound angiogenesis.

Poster Board Number: F-1172

THE ROLE OF MESENCHYMAL STEM CELL-CONDITIONED MEDIUM ON THE MIGRATION AND PROLIFERATION OF ENDOTHELIAL PROGENITOR CELLS

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Mesenchymal stem cells (MSCs) are multipotent stem cells, which have been regarded as potential cell sources for tissue replacement therapy. Although the ability of MSCs in restoring the damaged tissue by directed differentiation to generate specific cell types is still controversial, their role as a source of pro-angiogenic and anti-inflammatory cytokines is well established. There are several reports showing the positive effects of MSC-derived cytokines in ameliorating the symptom of ischemic heart disease and cerebrovascular disease in animal models. Recently, endothelial progenitor cells (EPCs) have been identified as an important regulator of vascular homeostasis and the major contributor of postnatal neovascularization. According to this, there might be an interaction between MSC-derived angiogenic cytokines and EPC function, which results in the neovascularization of ischemic tissues. In this study, the effect of cytokines secreted from postnatal tissue-derived MSCs on the proliferation, migration and invasion of EPCs were investigated and compared with those of bone marrow-derived MSCs using an *in vitro* model. The results showed that the conditioned medium from placenta-derived MSCs can promote the migration of cultured EPCs to about three times higher than other MSC sources. In addition, it appeared that some soluble factors secreted from bone marrow-derived MSCs can induce higher levels of EPC invasion compared with controls. In conclusion, this study demonstrated that the factors secreted by various MSCs might play different roles in the migration and invasion of EPCs. The factors involving in such process are currently being investigated.

Lung Cells

Poster Board Number: F-1174

RECAPITULATING THE HUMAN AIRWAY *IN VITRO* USING PNEUMACULTTM-ALI, A NOVEL MEDIUM FORMULATION FOR THE DIFFERENTIATION OF PRIMARY HUMAN BRONCHIAL EPITHELIAL CELLS AT THE AIR-LIQUID INTERFACE

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The human bronchial epithelium is a specialized structure designed to protect the lungs from noxious substances and perform a variety of immunomodulatory and secretory functions. Differentiation of primary human bronchial epithelial cells (HBECs) to a physiologically relevant mucociliated phenotype is achieved using air-liquid interface (ALI) culture methods with specialized media. Current ALI methods use semi-defined media formulations resulting in variable differentiation that often includes poor pseudo-stratification and minimal cilia or goblet cell formation. We have developed a robust, defined, serum-free medium, PneumaCult™-ALI, that consistently promotes the formation of pseudo-stratified, mucociliated cultures of HBECs. Primary HBECs from eight normal donors were obtained from commercial suppliers or the International Institute for the Advancement of Medicine (IIAM). Initial HBEC expansion was performed in a commercial medium (BEGM; Lonza). At p2 or p3, the medium was replaced with PneumaCult™-ALI for 16 h before cells were harvested and seeded onto collagen-coated porous culture inserts submerged in wells containing PneumaCultTM-ALI or control differentiation medium (BEDM; Lonza). Cells were maintained in submerged culture for 3 - 7 days until confluent then differentiated at the ALI by removing the apical chamber medium and replacing the basal chamber media every 48 h. Barrier function of ALI cultures was measured weekly by trans-epithelial electrical resistance (TEER), and diffusion of FITC-labeled dextran (4 kDa) and horseradish peroxidase (HRP; 44 kDa). At 21 - 28 d post-ALI, cultures were either 1) fixed and cross-sections stained using hematoxylin and eosin to show general morphology and periodic acid-schiff (PAS) to demonstrate mucous production or 2) processed for Western blot analysis for expression of goblet and ciliated cell markers. Separate cultures were treated for 14 d with IL-13 to examine mucous hyperplasia. Cultures grown in PneumaCultTM-ALI demonstrated stable TEER values for at least 24 weeks post-ALI induction (n=2). Only cells cultured in PneumaCultTM-ALI showed

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polarized barrier function whereby diffusion of FITC-dextran or HRP was limited in the apical to basal direction compared to the basal to apical direction. Cultures grown in PneumaCult™-ALI showed improved pseudo-stratification and mucociliated differentiation compared to control with increased expression of mucous (Muc5AC) and ciliated (cilia basal body) cell markers and the tight junction marker E-Cadherin (n=3-7). Treatment of PneumaCult™-ALI cultures with IL-13 resulted in mucous hyperplasia and increased Muc5AC expression. PneumaCultTM-ALI is a novel defined media formulation that provides consistent mucociliated differentiation of HBECs in ALI cultures resulting in morphological characteristics that are similar to that observed in the normal human airway. Furthermore, PneumaCult™-ALI cultures can elicit physiological responses to known stimuli, making our formulation a robust tool for *in vitro* lung epithelial cell research.

Poster Board Number: F-1175

INTERACTIONS OF ENDOTHELIAL CELLS WITH STEM AND PROGENITORS DRIVES CELL FATE AND ONCOGENESIS

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The adult mammalian lung is a complex, integrated system comprising numerous types of epithelial cells and surrounding stromal components. In the distal murine lung, Bronchioalveolar Stem Cells (BASCs) are an adult stem cell that responds to bronchiolar and alveolar cell injury and are hypothesized to give rise to adenocarcinoma. Alveolar type 2 (AT2) cells have long been proposed to serve as progenitors for alveolar epithelium. Epithelial-stromal interactions provide regulatory signals that maintain lung architecture and homeostasis. In lung cancer, such facilitate tumor progression. We hypothesized that functional interactions between stromal cells and stem/progenitor cells is critical for normal lung homeostasis, and for the production of tumors. By employing a matrigel-based 3D culture system, we compared the ability of five different stromal cell types to support BASC and AT2 activity in vitro. Serial passages revealed that normal mouse lung endothelial cells (MLuECs) enhanced the self-renewal ability of BASCs. The efficiency of colony formation of BASCs with MLuEC was determined to be 7.2%, by 7.6-fold greater than the one with lung fibroblasts (p=2.17E-5 and p=1.1E-4, respectively). BASCs gave rise to heterotypic colonies including that 18.3% of colonies look like bronchiolar structures containing cells positive for bronchiolar marker, CCSP, and 37.4% of colonies look like saccular/alveolar-like structures expressing alveolar marker, SPC, in the presence of MLuECs (p<0.01), whereas AT2 cells formed only alveolar-like structures. MLuECs facilitated BASCs to form bronchiolalveolar-like structures with 11.2% efficiency displaying branching morphogenesis (p<0.01). CCSP-positive bronchiolar cells were extended to generate alveolar clusters expressing SPC. Serial passages of a single colony of bronchiolalveolar-like structures showed characteristics of multipotent stem cells whose differentiation potential is dependent on the microenvironment. To examine multipotentiality of BASCs *in vivo*, we adapted the 3D matrigel assay to create an *in vivo* lung regeneration assay. Co-injection of dissociated bronchioalveolar-like colonies with MLuECs and matrigel subcutaneously showed the formation of bronchiolar- and saccualr/alveolar-like structures containing cells positive for CCSP, SPC and both in subQ plug suggesting multipotent activity of BASCs that is supported by MLuECs *in vivo*. Our assays with BASCs and MLuECs are the first to reveal heterotypic colonies that recapitulate distal lung epithelial cell types providing evidence that BASCs serve as stem cells for distal bronchioles and alveoli. Based on the result that MLuECs regulate lung stem cell activity, we examined whether MLuECs support lung tumor cell growth. Kras-driven lung tumor cells do not grow when injected into nude mice subcutaneously, but could grow when injected into trachea of the lung, suggesting requirement of tumor microenvironments. Co-injection of tumor cells with MLuECs revealed subcutaneous tumor cell growth indicating that endothelial cells support the activity of tumor cells as well as normal stem cells. Our studies define critical interactions between the stromal and stem/progenitor cells for lung homeostastis and cancer development.

Poster Board Number: F-1176

HUMAN LUNG STEM CELLS AND THEIR REGULATION BY P38A MAPK/MIR-17-92 CROSSTALK

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The existence and nature of human lung stem cells remains controversial, despite potential implications for both lung homeostasis and tumorigenesis. Research is now focussing on the connection between stem cells and cancer. The stress-activated protein kinase p38α plays a central role in lung homeostasis, regulating self-renewal and differentiation of putative human lung stem/progenitor cells (HLSCs). Absence of p38α leads to increased and accelerated responses to oncogenes, inducing earlier and more aggressive lung tumorigenesis. We have isolated adult human lung cells, based on Lgr6/E-Cadherin expression, which culture indefinitely and can be considered as stem/progenitor precursors of alveolar epithelium. Optimised protocols enable in vitro differentiation and transformation of HLSCs, observing a matrix-directed lung cell specification in a variety of substrates (Matrigel, Fibronectin and Laminin). *In vivo* assays to investigate cellular differentiation and self-renewal potential under physiological conditions included murine kidney capsule implants, which provided conditions promoting tissue differentiation. We showed that implanted HLSCs differentiate into various lung cell types *in vivo* and are morphologically similar to the lung, whilst also expressing lung tissue specific markers. The contribution of HLSCs to repair of both human lung explants in vitro and murine lung tissue *in vivo* was also shown following bleomycin-induced injury. Cytokine and growth factor release, that are central to tissue remodelling, has also been demonstrated. We have previously shown that p38α regulates homeostasis of mouse lung stem cells. We now show that stable p38 knockdown HLSCs have similar properties to p38α -/- mouse lung stem cells, with an increase in proliferation and defective differentiation. p38α loss alters lung tissue specific marker expression, which may relate to a defective response to the different matrix proteins used. HLSCs lacking p38α showed upregulated expression of the miR-17-92 microRNA cluster. The miR-17-92 cluster has been shown to have an opposite role to p38α, acting as a suppressor of lung differentiation while promoting lung cell proliferation. The role of miR-17-92 microRNAs in p38α-dependent regulation of lung homeostasis and the effect of microRNA-deregulation on p38α activity in HLSC homeostasis as well as in lung cancer were investigated. HLSC homeostasis was shown to be dependent on the balance between p38α and miR-17-92 cluster signalling. A model in which miR-17-92 expression may be repressed by p53 is proposed. p53 can be activated via serine 20 phosphorylation by the p38α mediator kinase, MAPKAPK2, repressing the miR-17-92 promoter and transcription. Predicted targets of miR-17-92 include the lung differentiation transcription factors c/EBPα and Gata-6, and we demonstrated their direct regulation by miR-17-92 in HLSCs. Downregulation of those factors correlated with the loss of some lung tissue specific markers and upregulation

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of stemness markers, together with misexpression of integrins. This work showed the role of the p38α pathway in the differentiation and self-renewal of newly characterised HLSCs and the balance between p38α and miR-17-92 signals. Defects in this control may result in disease (e.g. cancer) or defective regeneration (e.g. lung fibrosis). We have found new markers to detect and isolate human lung stem cells, providing a new step forward in the search for potential cell regenerative therapies.

Poster Board Number: F-1177

THE STUDY OF ALVEOLAR CELLULAR LINEARAGE DIFFERENTIATION INTO TYPE-I PNEUMOCYTES *IN VITRO*

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The lung is the essential respiration organ in all air-breathing animals. The major part of lung structure is gas-exchanging air-space (alveolar sacs). The alveolar sacs consist of many functional gas-exchanging units, named alveoli. There are approximately 300 million alveoli in adult lung. The type-I and type-II pneumocytes contribute to the maintenance of the epithelial layer for alveoli formation. Type-I pneumocytes are large, thin cells stretched across a large surface area, and cover a majority of the alveolar surface area (>95%), and are responsible for gas exchange. However, the knowledge about Type-I pneumocyte was quite rare because the difficulty of purification of the Type-I pneumocyte in past, as well as, the main function of Type-I pneumocyte: blood-air barrier for oxygen transportation was also quite hard to study. Recently, Aqp-5, Caveolin-1 and T1α had been reported as surface markers to identify Type-I pneumocytes, respectively. In our study, we have developed a novel system that capable to isolate and culture lung stem/progenitor cells which could express pluripotency-associated transcription factors, NANOG, OCT4 and SOX2; and the cells have the ability to undergo terminal differentiation into Aqp-5+/ Caveolin-1+/ T1α+-Type-I pneumocytes in induction condition, which were examined by RT-PCR and immunofluoresce labeling. In time-lapse photography, the cells also showed morphological change from cuboid shape into large flatten squamous Type-I pneumocytes within diameter lager than 100 μm. By using microfluidic device, the biological function of Type-I pneumocytes has been investigated. In the device, the Type-I pneumocyte showed better ability of oxygen transportation/permeability than A549 and NIH-3T3. This data indicates that the Type I pneumocytes have not only expressed cellular specific markers for Type I pneumocytes, but presented the real gas-exchanging function within it. In our knowledge, this is the first *in vitro* model capable of demonstrating alveolar differentiation lineage, and the cells can be a new platform to investigate the function of Type-I pneumocyte and the detailed mechanism of oxygen transportation in alveoli.

Poster Board Number: F-1178

GENERATION OF INDUCED PLURIPOTENT STEM CELLS FROM PATIENTS WITH IDIOPATHIC PULMONARY FIBROSIS

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Idiopathic pulmonary fibrosis (IPF) is a debilitating disease that is characterized by chronic, progressive, irreversible fibrosis of the lung interstitium. There is no treatment for this devastating disease except lung transplantation. The etiology of IPF is unknown and as it is usually a disease of older age, it is likely the result of a complex interplay of genes and environment. The bleomycin rodent model that is used to study IPF results in fibrosis that resolves after a few weeks, and the field is therefore lacking a relevant model of IPF. Induced pluripotent stem cells (iPSC) have been used to model other complex diseases including Alzheimer's disease, Lou Gehrig's disease, Rett Syndrome, and Duchenne Muscular Dystrophy, where genes and environment play a role in disease pathogenesis. But iPSC have not yet been used to model IPF. We therefore sought to create a human model of IPF and better understand reprogramming of fibroblasts from fibrotic as compared to normal lungs. We generated iPSC lines that were reprogrammed from IPF and healthy lung fibroblasts under good manufacturing practices (GMP) conditions. Biopsy samples from the skin and lungs of IPF patients were obtained at the time of lung transplantation. These, along with healthy donor lung fibroblast controls from the lung transplant donor, were then used to generate iPSC lines. To create the iPSC lines, we used the human STEMCCA lentiviral cassette containing four transcription factors; OCT4, SOX2, cMYC, and KLF4, to reprogram the fibroblasts. We confirmed pluripotency of the iPSC lines by teratoma formation as well as by immunofluorescence for pluripotency markers: OCT4, SOX2, TRA1-81, and NANOG. In addition, all iPSC lines had a normal karyotype. We performed RNA-seq and BS-seq to examine the transcriptome and methylome of the fibroblasts from IPF lungs, healthy lungs and the iPSCs generated from them in order to more fully understand the similarities and differences between the diseased and healthy fibroblasts. Preliminary RNA-seq analysis of the iPSC lines showed an upregulation of pluripotency genes and a downregulation of genes associated with fibroblast differentiation, further confirming that the reprogramming process was successful. The RNA-seq data also showed an upregulation of genes associated with DNA repair, aging and senescence in the diseased fibroblasts that was also present in their derived iPSC. In order to understand more about IPF and its multifactorial etiology, we are also using the iPS cells to model the disease and understand the process leading to fibrosis. Our work has shown that it is possible to generate iPS cells out of lung fibroblasts from patients with idiopathic pulmonary fibrosis and will likely provide new insights into the pathogenesis of the disease that could lead to novel therapeutic strategies.

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Epidermal Cells

Poster Board Number: F-2001

DYNAMICS OF CULTURED HUMAN EPIDERMAL KERATINOCYTE STEM CELLS

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Cultured human epidermal keratinocyte stem cells (holoclones) progressively lose their proliferative capacity during serial cultivation to become cells with restricted growth capacity (paraclones), a phenomenon termed clonal conversion. Clonal conversion is irreversible under normal conditions and will ultimately lead to the termination of the culture. Clonal conversion is accelerated by suboptimal culture conditions, serail cultivation and aging. The maintenance of keratinocyte stem cells in suitable culture conditions is indispensable for successful transplantation, and yet behaviors of cultured keratinocyte stem cells have been poorly elucidated. In this study, we analyzed the dynamics of keratinocytes with different growth potentials. Holoclones and paraclones significantly differed in their immediate response to EGF that induced a rapid expansion of colony size in holoclone-derived colonies whereas it induced a significant reduction in colony size in paraclone-derived colonies. The distinct response to EGF was due to the difference in the organization of their actin filaments between holoclones and paraclones. We also found the difference in spontaneous migration of keratinocytes between holoclone- and paraclone-derived colonies. These results indicate that cultured keratinocytes act differently depending on their growth capacity, suggesting that keratinocyte stem cells can be identified with microscopic observation in culture.

Poster Board Number: F-2002

TOWARDS HES CELL-BASED THERAPY FOR SKIN REGENERATION

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The epidermis is a highly specialized epithelium that has evolved to provide a stable environmental barrier to prevent dehydration, resist mechanical and chemical stress and participate in immune responses. However, despite its remarkable stability, epidermis is amongst the most dynamic tissues in human body, which allows for continuous tissue regeneration. This function is supported by epidermal stem cells, which are responsible for constantly renewing the epidermis and generating the skin barrier. However, skin regeneration can be severely compromised in degenerative skin conditions such as Epidermolysis Bullosa (EB). EB is a group of blistering skin disorders resulting from mutations in one of 14 different genes encoding protein components of the cutaneous basement membrane zone (BMZ). These mutations affect the organization of BMZ, causing the epidermis to separate from the dermis. Constant blistering is likely to deplete the pool of epidermal stem cells, therefore the ideal cell therapy would have to be aimed at replenishing the endogenous stem cell pool. The overall goal of this work has been to utilise human embryonic stem cells (hESCs) for the eventual derivation of epidermal cells with a significant proliferation potential that can potentially be used for cell-based therapy for EB. It was hypothesized that by mimicking the natural microenvironment under which epidermal stem cells are developed and maintained *in vivo*, human embryonic stem cells can be successfully directed to epidermal cell fate. To achieve this goal organotypic techniques utilizing de-epidermized dermis (DED) were employed. Short stretches and cysts resembling stratified squamous epithelia were detected in DEDs. These structures stained positively for Keratin 14 protein expression and were surrounded types IV and VII collagen deposited by hESCs. However neither continuous epithelial layer nor discrete basement membrane was observed, suggesting that the stimuli provided were insufficient for a complete epithelia formation from hESCs. Therefore, we hypothesized that by pre-differentiating hESCs into primitive epidermal cell lineages in vitro prior to seeding onto DEDs, the efficiency of epithelial formation could be significantly improved. Consequently, various differentiation techniques have been devised and investigated. The most successful approach relied on native de-cellularized extracellular matrix produced by normal human dermal fibroblasts (HDF-ECM) as a substrate for differentiation. The putative epidermal precursors have been characterized for the expression of epidermal stem cell markers by immunofluorescence, quantitave PCR and flow cytometry. The proliferative and clonogenic potential was also evaluated by the means of several assays. Large epithelial-like sheets positive for Keratin 14 and p63 transcription factor expression were observed. On subculture these cells retained their morphological and immunocytochemical characteristics for up to 5 passages and could be successfully cryo-preserved and recovered. The results suggest that HDF-ECM can successfully encourage differentiation of hESCs towards highly proliferative keratinocyte precursors (pKPCs) by mimicking the natural environment of epidermal cells, i.e. basement membrane of human skin.

Poster Board Number: F-2003

HAIR INDUCTION BY TRANSPLANTATION OF HUMAN FOLLICULAR CELLS , DERMAL PAPILLA OR THEIR COMBINATION IN NUDE MICE

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Human diseases cause to miss organ function so we need to replace lost or injured organ by regrowth of a new organ or tissue. Stem cell research provides the promise of developing treatment for debilitating diseases. Skin is an excellent and first organ in which stem cells have used to replace damaged epidermis on burn patients successfully. Now skin biologists have begun to profit skin stem cells in other skin diseases. One of the most important skin diseases that many of patients repine about it is hair loss (alopecia). The request for drugs that alter hair growth and appearance has led to a multibillion-dollar industry, yet few drugs that are effective for these purposes are available. Although several study has suggested using dissociated hair precursor cells to have *de novo* formation of hair follicles in vivo but no research existed that show potential of adult human dermal papilla and bulge stem cell(hair follicle cells) to create new hair. This study aimed to identify efficient, minimally invasive and economical follicular cells transplantation Procedure for use in clinical settings. Toward this end two types of human follicle cells directly isolated were cultured for up to 4 weeks then the expression of bulge stem cell marker (CD200,CD29,CD34)and dermal papilla marker Integrin α9 were tested by flowcytometry. To determine which of them- bulge cells (B) , dermal papilla(DP) or their combination- are necessary for production hair, labeled cells(dermal papilla with PKH26=RED and PKH26=GREEN) were

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transplanted into Nude Balb/c mice(4-6 weeks of age),and hair follicle regeneration was evaluated histologically.Total hair created, quality of hairs and hairs survival time was recorded every week. Isolated bulge cells showed high level of CD200 expression and low level of CD29 and CD 24 expression and DP cells showed high level of Integrin α9. After 4 weeks of injection in control group (media) 30 hair follicles surrounded by sebaceous glands without maturation towards terminal hair and shaft was observed however in DP group 102 hair follicles in average were detected in consequence maturation stages and most of them containing hair shaft. In DP+B group thousands hair follicles in average were determined most of hair follicle in early anagen phase. These hairs remained until 10 week in all of groups after injection. In addition to the transplanted PKH (green and red)-labelled cells were detected in all labeled groups. Experiments in B group are going. In conclusion our these results suggest that direct contact of epithelial and dermal components is efficient to produce numerous hairs. Another major advantage of this method is developing a simplified procedure that can be useful to test the ability of candidate cells to produce hair.

Poster Board Number: F-2004

MODULATION OF EPITHELIAL PROGENITORS BY VIRAL ONCOGENES

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Human papillomaviruses (HPVs) are clinically important viruses, which ubiquitously infect stratified epithelia of humans. Notably, infection with high-risk HPVs can lead to malignancy including cervical cancers. Cervical cancers arise frequently from the transition zone, the niche for cervical progenitors. The main viral oncogenes E6 and E7 have been shown to play essential roles both in the viral life cycle as well as the development and maintenance of cancer, but their specific effects on the progenitors of the tissue are not known. However, both E6 and E7 have been shown to impinge on cellular factors critical for stem/progenitor behavior. Using mouse models that target expression of the viral oncogenes to the basal layer of statified squamous epithelia we evaluate the effect of viral oncogene expression on progenitor cell homeostasis. Results from this study will enrich our understanding of the modulation of progenitor compartments by infectious agents, and the role of such effects in ensuing pathology.

Poster Board Number: F-2005

STABILIZATION OF FIBROBLAST GROWTH FACTOR-1 IMPROVED RADIATION-INDUCED HAIR FOLLICLE DYSTROPHY WITH THE MAINTENANCE OF HAIR FOLLICLE STEM CELLS.

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Radiation-induced hair loss is a clinically important topic because it influences the patients negatively to decide to choose radiation therapy. Several fibroblast growth factors (FGFs) have been found to be able to protect against radiation damage. In particular, FGF1 is able to activate all of the known tyrosine kinase FGFR subtypes and is responsible for many biological effects, so that the wide spectrum of FGF1 activity was expected to be advantageous to treat radiation injuries. However, FGF1 has poor thermal stability and a relatively short half-life *in vivo*; therefore, the structural instability of FGF1 limits its potential for practical use. Hence, an FGF1 triple mutant Q40P/S47I/H93G was created as the most stable and active FGF1. Q40P/S47I/H93G had the same receptor specificity as wild-type FGF1 and the in vitro mitogenic activity of Q40P/S47I/H93G in the absence of heparin was at least 10 times stronger than wild-type FGF1. In addition, Q40P/S47I/H93G had the potent protective effects against radiation-induced intestinal damage and prolonged mouse survival after total body irradiation (TBI) because of the repair of intestinal damage. To evaluate its effects against radiation-induced hair follicle damage, a portion of the dorsal skin, harboring uniform telogen phase hair follicles, was depilated from 7-week-old male BALB/c mice to induce the anagen phase of the hair growth cycle. Then, these mice received TBI with gamma-rays 6 days after depilation to induce hair follicle dystrophy. Under these conditions, hair could grow after irradiation at 4 Gy, but hair growth was extremely delayed at 6 Gy, and K15 positive stem cells decreased in anagen hair follicles in a radiation dose-dependent manner. When Q40P/S47I/H93G or wild-type FGF1 in the absence of heparin was administered intraperitoneally 24 h before irradiation, Q40P/S47I/H93G treatment significantly decreased TUNEL-positive cells in hair bulbs 24 h after TBI at 12 Gy compared with wild-type FGF1. Treatment with Q40P/S47I/H93G noticeably decreased the appearance of activated caspase 3-positive cells in hair bulbs in a dose-dependent manner 8 h after irradiation, but not with wild-type FGF1. In addition, Q40P/S47I/H93G enhanced BrdU incorporation in hair bulbs after irradiation. Q40P/S47I/H93G maintains the number of K15 positive stem cells in the bulge regions of hair follicles 3 days after TBI at 4 Gy, although wild-type FGF1 could not prevent the decrease of K15 positive stem cells. Moreover, K15 positive cells increased in the lower outer sheath after Q40P/S47I/H93G treatment. These findings suggest that the structural stability of FGF1 can increase the capability for self-renewal of stem cells, resulting in the maintenance of stem cells in their differentiation. Therefore, Q40P/S47I/H93G may be a candidate for the treatment or prevention of hair loss after radiation therapy.

Poster Board Number: F-2006

WNT/BETA-CATENIN SIGNALING REGULATES MELANOCYTE STEM CELL DIFEFRENTIATION IN UVB-INDUCED EPIDERMAL PIGMENTATION.

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[Background and Purpose] Melanin produced by melanocytes is responsible for skin and hair pigmentation. Recent studies have demonstrated that melanocyte stem cells, the origin of melanocytes, locate in the bulge area of hair follicles. It is reported that melanocytes in hair bulbs are supplied being synchronized with hair growth cycles from melanocyte stem cells. However, the relationship between epidermal melanocytes and melanocyte stem cells is not yet clearly understood. In our study, we specifically analyzed the change in melanocyte stem cell behavior caused by UV-B irradiation and the role of Wnt/β-catenin signaling in order to elucidate the mechanism of the differentiation of melanocyte stem cells into epidermal melanocytes. [Results and Discussion] UVB light (100 mJ/cm^2, 3 times/week, four weeks) was irradiated on the back of

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F1 mice of HR x De mice (melanin-containing hairless, 7-week old, male). Tyrosinase-related protein 1 (Tyrp1)-positive melanocytes appeared transiently in hair follicles three days after the initial irradiation. Seven days after the initial irradiation, Tyrp1-positive melanocytes were increased in the epidermis. 14 days after the initial irradiation, skin pigmentation became macroscopically visible. Concurrently, significant acceleration of expression of Wnt7a and an increase in the number of nuclear β-catenin-positive cells in hair follicles were noted. When IWR-1 (inhibitor of Wnt/β-catenin signal) or siRNA for Wnt7a were administered to the mice, the increase in Tyrp1-positive melanocytes, which was caused by UVB irradiation, was suppressed. These findings suggested that melanocyte stem cells differentiated in hair follicles and migrated into epidermis in response to Wnt/β-catenin signaling induced by UVB irradiation. In the analysis of time-dependent change in skin pigmentation, although the skin pigmentation once disappeared four weeks after the completion of irradiation, solar lentigo-like pigment spots were noted 12 to 32 weeks after the completion of irradiation. At the same time, an increase in Tyrp1-positive melanocytes and changes in Wnt/β-catenin signaling-related gene expression were observed. These result suggested that an abnormal regulaiton of Wnt/β-catenin signaling due to UV-B irradiation may be associated with development of solar lentigines. Based on the above results, it was considered that the differentiation of melanocyte stem cells into epidermal melanocytes is closely related with Wnt/β-catenin signaling. And various kinds of pigment disorders including solar lentigines are caused by the abnormal regulation of the signaling. We will conduct further investigations on the mechanism of the differentiation of melanocyte stem cells into epidermal stem cells.

Poster Board Number: F-2007

PARACRINE TGF-B SIGNALING COUNTERBALANCES BMP-MEDIATED REPRESSION IN MOUSE HAIR FOLLICLE STEM CELL ACTIVATION

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Regeneration of adult tissue relies on the activation of quiescent stem cells (SCs) regulated through multiple signals, which either stimulate or inhibit SC proliferation. Hair follicles (HFs) in mouse skin offer a unique opportunity to explore this process. Throughout adult life, they undergo dynamic, synchronized cycles of degeneration, resting, and regeneration phases and produce one hair in each cycle. In the HF, the SCs locate within the specialized microenvironment, or bulge niche, which is composed of epithelial SCs in the bulge and secondary hair germ, and underlying mesenchymal cells called dermal papilla (DP), as well as melanocytes, smooth muscle fibers, sensory neurons, and adipocytes. During resting phase, which can last for months, BMP2/4/6 and FGF18 signaling, both extrinsic and intrinsic maintain SC quiescence mainly by CDK4 repression through Nfatc1. HF regeneration begins when communication between quiescent SCs and underlying DP generates sufficient activating cues to overcome BMPs/FGF18 repressive signals in the niche, which results first in the proliferation of more closely situated SCs in hair germ and subsequently the more distant SCs in the bulge. Wnts, BMP inhibitory factors, and FGF7/10 are known as activating cues resulting from DP-SCs crosstalk. It is likely that the combination of growth factors and crosstalk among the intracellular signaling pathways defines SC behaviors. However, exactly how the transition from quiescent to active state comes about remains unclear as do the downstream events that drive SCs into a tissue regenerative state. TGF-β signaling induces Smad2/3-mediated gene expressions and functions in tissue morphogenesis, homeostasis, and cancer formation by regulating diverse biological processes including proliferation, differentiation, and extracellular matrix production. Interestingly, although TGF-β is a growth inhibitor for most epithelial cells, it has multiple and often opposing effects depending on the tissue and the type of cells. Here we uncovered a hitherto unrecognized paracrine DP transmitter, TGF-β2, which activates transient Smad2/3 signaling in HFSCs at the transition from quiescent to tissue regenerative state. This signaling is critical: HFSCs that cannot sense TGF-β exhibit significant delays in HF regeneration, whereas exogenous TGF-β2 sitmulates HFSCs *in vivo* and in vitro. By engineering TGF-β- and BMP-reporter mice, we showed that TGF-β2 signaling antagonizes repressive BMP signaling in HFSCs. TGF-β and BMP pathways utilize a common transcription factor Smad4, however, BMP repression by TGF-β in HFSCs was not through competition for limiting Smad4-coactivator. Rather, our microarray, molecular, and genetic studies unveiled Tmeff1 as a direct TGF-β2/Smad2/3 target gene, expressed by activated HFSCs and physiologically relevant in restricting and lowering BMP threshold in the niche. Furthermore, HF regeneration in epidermis-specific *in vivo* knockdown mice underscored that Tmeff1 is a mediator of SC activation at the transition from quiescent to regeneration phase. Our study also demonstrated that even in the same epidermal cells, responses to TGF-β are different in HFSCs and keratinocytes. As an underlying mechanism, inherent BMP signaling in HFSCs was required for the TGF-β's antagonizing effect. Connecting BMP activity to an SC's response to TGF-βs may explain why these signaling factors wield such diverse cellular effects.

Poster Board Number: F-2008

USING AN INDUCIBLE TET-ON ANIMAL MODEL TO STUDY THE RESPONSES OF MOUSE STEM CELL SUBPOPULATIONS IN HAIR FOLLICLES TO TGF-BETA SIGNALING

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Epidermal stem cells residing in different locations in the skin continuously self-renew and differentiate into distinct cell lineages to maintain skin homeostasis during postnatal life. Murine epidermal stem cells located at the bulge/secondary hair germ region are responsible for replenish the hair lineage, while the stem cells at the junction between the infundibulum and hair follicle (isthmus) fuel into interfollicular epidermis (IFE) and sebaceous glands (SG). TGF-β signaling transduced from ligands (TGF-β1-3) binding to type II (Tgfbr2) and type I (Alk5) receptors, which induces phosphorylation of Smad2/3, complexes with Smad4, and translocates into the nucleus to activate downstream target genes. In vitro cell culture and *In vivo* animal studies have implicated TGF-β signaling in the maintenance of epideramal and hair cycle homeostais. Mice with epithelial-specific deletion of Tgfbr2 and Alk5 die of cleft palate, thus dampened studies to assess the role of TGF-β receptors in postnatal hair cycle and skin homeostasis. To circumvent the perinatal lethality due to TGF-β receptor loss, we employed an animal model in which Cre/LoxP system and rtTA/TRE system were combined to allow inducible and reversible attenuation of TGF-β signaling. We have previously developed a Tgfb3-Cre mouse line which induces gene recombination in hair follicle lineages and suprabasal layer of the epidermis, and we further applied this mouse line in triple-transgenic animals (TTG) carrying additional two transgenes Rosa-rtTA-IRES-EGFP and tetO-dominant negative Tgfbr2 (dnTbr2). TTG animals administered with doxycycline

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displayed robust expression of dnTbr2 and inhibition of Smad2/3 phosphorylation in Tgfb3-Cre-expressing cell lineages. Using this animal model, we have analyzed the role of TGF-β signaling in distinct phases of the hair cycle. Transient abrogation of TGF-β signaling does not prevent catagen progression; however, it induces a blockade in anagen re-entry and an incomplete hair shaft development as well as results in aberrant epidermis and sebocyte lineage differentiation of isthmus stem cells. Moreover, ablation of TGF-β signaling also leads to an increase of apoptotic cells in the secondary hair germ and bulb matrix cells, and blocking of TGF-β signaling in the HF stem cells abolishes their colony-growing ability in in vitro colony forming assays. These data strongly suggest that TGF-β signaling plays an important role in regulating the activity of distinct epithelial stem cell populations.

Cardiac Cells

Poster Board Number: F-2009

NEW METHOD FOR CONTROLLING SIZE OF HUMAN CARDIOSPHERE AND ITS CARDIOVASCULAR DIFFERENTIATION POTENTIALS

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Background: Cardiospheres are a promising cell source to prevent MI. However, cardiospheres are known to have some limitations in terms of controlling sizes, maintaining multipotency for cell transplantation so far, because of its formation in irregular sizes. Excluding large clusters of cardiospheres before cell transplantation leads smaller number of disaggregated single cells from spheres or demands laborious experimental steps to dissociate into smaller sizes.Here, we investigated a new method that enhances their potency, enriches cardiospheres and controls sphere sizes for transplantation. Methods and Results: We used secondary cardiosphere to control sizes by applying 4 different materials for experiments; poly-D-lysin, petri dish, hanging drop method and using AggreWell™ plate. The mRNA expression of Oct4 was higher when cardiospheres are formed in an AggreWell™ plate than plated on poly-D-lysin coated dishes (2.191 folds) or with hanging drop method (1.674 folds). The expression of Nanog was also the highest when cardiospheres were cultured in AggreWell™ plates. The size of cardiospheres using AggreWell™ plates and hanging drop method was no bigger than 100 μm in relatively homogeneous size and shape while spheres formed using poly-D-lysin and petri dish were heterogeneous in size and shape. Moreover, comparatively short period of time was required for maturation of cardiosphers when produced on AggreWell™ (about 24hrs) compared with on poly-D-lysin-coated dish (about 72hrs). But the weak point of using AggreWell™ plate is the high costs compared to other materials. The results of comparison between 4 different materials are summarized in the table. We found differentiation potentials of human cardiospheres cultured on AggreWell™ plates to cardiovascular lineage in myocardial infarction model of nude mice, along with investigating the mechanism of generation of human cardiospheres by using chemical blockers, blocking antibody. Conclusions: These results suggest that small and homogenous size in cardiospheres are important to induce large amount of cells to engraft with greater efficiency.

Poster Board Number: F-2010

HUMAN ATRIAL APPENDAGE: A POTENTIAL SOURCE FOR GENERATING TRANSGENE FREE INDUCED PLURIPOTENT STEM CELL DERIVED CARDIOMYOCYTES

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Left atrial appendage (LAA) is typically a byproduct of coronary artery bypass surgery in patients with myocardial infarction. LAA is an attractive source of cardiac fibroblasts for reprogramming into induced pluripotent stem cells (iPSCs). We reprogram human atrial derived fibroblasts using non-integrating episomal plasmids, study cardiac differentiation and investigate ion channel characteristics of hiPSC-derived cardiomyocytes. Our human iPSCs expressed markers of pluripotency and generated teratomas. They also readily formed embryoid bodies and differentiated into cardiomyocytes with comparable efficiency to well established human embryonic stem cells (hESC). Temporal gene expression of the hiPSCs derived cardiomyocytes indicated that differentiation was initiated by increasing expression of cardio/mesodermal markers, followed by cardiac-specific transcription factors, structural and ion channel genes. The cardiomyocytes showed characteristic cross-striations of structural proteins like alpha actinin and troponin T, confirming their cardiac ontogeny. Microelectrode array recordings further established the electrotonic development of an excitation-contraction coupling mechanism that responded pharmacologically to active agents. They showed chronotropic dose-response to isoproterenol and carbamycholine that closely resembles functioning myocardium. These results for the first time demonstrate that atrial appendages, a surgical "waste" could be efficiently utilized for generating hiPSCs and differentiating into functional cardiomyocytes that are potentially useful for autologous cardiac cell therapy.

Poster Board Number: F-2011

NOVEL SMALL MOLECULES INDUCE ROBUST AND HIGHLY EFFICIENT CARDIAC DIFFERENTIATION FROM HUMAN ES AND IPS CELLS

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The cardiomyocytes derived from human ES/iPS cells are important for drug discovery screening of a pharmacological QT prolongation test and a cardiac cell transplantation therapy. We established High Throughput Screening (HTS) system searching for compounds promoting cardiac differentiation of ES cells. Using this HTS system, we identified one novel type small molecule KY02111. This KY02111 compound induced cardiac differentiation with extremely high efficiency (90 - 96 %). Then, the KY02111-induced cardiomyocytes expressed many cardiac marker genes (aMHC, bMHC, cTnT, NKX2.5, αActinin) and cardiac channel genes (HCN4, HERG, KCNQ1) remarkably. The electrophysiological study showed drug induced QT prolongation and functional maturation of these cardiomyocytes, consistent with gene expression data. Moreover, KY02111 had robust effects to induce cardiac differentiation in several ES/iPSC lines regardless of species (mouse, monkey, human). These results suggest that our HTS system is a successful screening model using ES/iPSCs, and KY02111 is a very useful compound for producing

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functional cardiomyocytes from human ES/iPSCs. This new cardiac differentiation method is expected for drug screening and cell therapy of heart disease.

Poster Board Number: F-2012

DETERMINATION OF APPROPRIATE DIFFERENTIATION STAGE OF HUMAN INDUCED PLURIPOTENT STEM CELL-DERIVED CARDIOMYOCYTES FOR DRUG ASSESSMENT

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Human induced pluripotent stem cell-derived cardiomyocytes (hiPS-CMs) have received much attention because of their potential for drug efficacy/safety screening. While previous studies have demonstrated electrophysiological analyses using hiPS-CMs at days 20-40 of differentiation, the functional and morphological characters in hiPS-CMs at day 60 or a later stage of differentiation and comparison with those in adult heart have not been fully elucidated. In the present study, to rectify this deficiency of information, we profiled the functional and morphological characteristics with global gene expression in hiPS-CMs at days 50-60 and 80-90 of differentiation and compared them with those at days 20-30 of differentiation or with adult ventricle tissue and determined the appropriate developmental stage for the assessment of drug efficacy or toxicity. Isoproterenol, a β-adrenergic compound, increased the beating rate in hiPS-CMs at days 20-30, 50-60, and 80-90 of differentiation (d30CMs, d60CMs and d90CMs, respectively) and microelectrode array analyses using calcium, potassium and sodium channel blockers indicated that the electrophysiological properties related to these ion channels were activated at all differentiation stages in a comparable magnitude. Ultrastructural analyses of hiPS-CMs at each stage using transmission electron microscopy showed that the morphological properties such as myofibrils in d60CMs and d90CMs were equivalent and more mature in their form and distribution than those in d30CMs. Analysis of the motion vectors in moving cells showed that the velocity of contraction, an index of cardiac contractile function, was higher in d90CMs than in d30CMs and the contractile function of d90CMs was most mature in the 3 stages examined. Interestingly, we found a few rod-shaped cardiomyocytes, which had much higher velocities of contraction than the sphere-shaped cells in hiPS-CMs at each stage. Global gene expression analyses demonstrated that many essential cardiac gene expressions related to development of the heart and morphologic components had already developed at day 30 of differentiation. Some gene expression levels such as myosin-binding protein C and S100A1, which are related to the myofilament and sarcoplasmic reticulum (SR), respectively, were increased with maturation of the morphological components and contractile function, and their gene expressions might be good markers to check morphological and functional maturation. Our profiling revealed that d30CMs are usable for basic screening such as functional and electrophysiological properties related to the β-adrenergic pathway or ion channels. d60CMs are also appropriate for morphological assays based on their structural maturity. For the assay of contractile function integrated by sub-cellular component such as SR and filaments, d90CMs are the most suitable hiPS-CMs among the 3 stages.

Poster Board Number: F-2013

A SMALL MOLECULE THAT DIRECTS CARDIAC PACEMAKER CELL DIFFERENTIATION FROM HUMAN AND MOUSE INDUCED PLURIPOTENT STEM CELLS

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Small molecules harbor the potential to drive the fate of pluripotent cells towards certain lineages. Embryonic stem (ESC) and induced pluripotent stem (iPSC) cells are attractive sources for *ex vivo* generation of cardiomyocytes. These systems are well-suited for developmental studies, high throughput drug screenings and cell replacement approaches. We have previously shown that the activation of calcium-activated potassium channels (SKCas) via the small molecule 1-EBIO (1-Ethyl-2-benzimidazolinone) leads to an induction of mesodermal differentiation and an enrichment of cardiac pacemaker cells. To date, these findings were restricted to mouse embryonic stem cells, thus limiting a broad applicability. It is obvious that this strategy could be a powerful approach for clinical and research applications. Therefore, we have analyzed several critical parameters using pluripotent stem cells from different sources and species in a systematic approach including a translation to the human system. We assessed SKCa-function via the small molecule 1-EBIO in terms of cardiac differentiation under various culture conditions using pluripotent ESCs and iPSCs and provide the following results: (i) an easy to handle and highly efficient differentiation assay, (ii) with broad application potential, (iii) the generation of virus-free human iPS cells as a potential source for patient-specific iPS cells and (iv) transfer of the SKCa activation assay to the human system. In addition, we present more mechanistical data and are now able to generate a pathway model. We assume, that in SK channel activity exposed embryonic stem cells, SK channels bind to CaM, probably to Calm1 and Calm2. The recruitment of CaM leads to activation of the Ras/Raf/MEK/ERK pathway. In addition, the CamkII (CamkIIg or CamkIId) has an indirect effect on ERK. MKP could have a Ca2+ dependent negative effect on ERK1 as a kind of fine. Therefore, the sustained phase of ERK1 activation could lead to ES cell differentiation towards the cardiac lineage. In summary, our data clearly demonstrate the generation of SKCa-induced cardiac pacemaker cells from ESC and iPSC and overcome specific limitations for potential applications. With our system using human iPSCs we further identify new strategies for the generation of autologous cells for cell replacement applications, thereby, providing a key milestone on the path to making cardiac subtypes. Finally we shed led light on a potential mechanism mediating SK channel driven cardiogenesis from pluripotent stem cells.

Poster Board Number: F-2014

UNIFORM SPHERES OF HUMAN PLURIPOTENT STEM CELL-DERIVED CARDIOMYOCYTES FOR EXTENDED *IN VITRO* TOXICITY TESTING AND PHENOTYPIC ASSAYS.

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The availability of human cardiomyocytes from pluripotent stem cells has provided opportunities for more predictive toxicology testing by avoiding the need for interspecies extrapolation. Human cardiomyocytes from non-genetically modified pluripotent stem cells can offer a physiological relevant source. These car-

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diomyocytes appear, in differentiated cultures, as multicellular aggregates which are inherently heterogeneous in shape, size as well as electrophysiological characteristics. However, conformity and repeatability of molecular, morphological and functional characteristics is required for a low signal to noise ratio, essential for screening campaigns. To achieve this goal of homogeneity for large scale assays, human embryonic stem cell-derived cardiomyocytes were reformed into near-identical spheres, hES-CMC UniSpheres™. These were evaluated for consistency in shape, size, molecular signature and electrophysiological properties within batch, across batches and during in vitro maturation. The hES-CMC UniSpheres™ were large enough to manipulate without the need for a microscope, and robust enough to allow multiple transfers between measurement platforms and culture environments. The hES-CMC UniSpheres™ demonstrated remarkable homogeneity in size, both within and across batches. Furthermore, the absence of the overgrowth typically associated with mix population cultures negated the need for passaging. The expression of key cardiac markers, such as ACTC1, TNNT2, MYL2, MYH6, MYH7, ANF, HCN1, KCNa5, Sarcolipin, KCNJ2 and KCNH2 were stable for at least 40 days. Electrophysiological studies showed that the range of beat frequencies was substantially reduced compared to the original clusters. Beating rates remained stable during monitoring and were consistent over extended periods in vitro. Furthermore, hES-CMC UniSpheres™ released cardiac troponin in a concentration dependant manner after exposure to doxorubicin. In summary, hES-CMC UniSpheres™ exhibited extended in vitro longevity, without the loss of tissue-specific morphology and function. In addition, the hES-CMC UniSpheres™ could withstand multiple transfers to non-labelled assay platforms. This stability and robustness can facilitate long term studies with repeated measures, such as multiple exposure or chronic toxicity testing. Furthermore, release of established biomarkers with direct relevance to human health, such as cardiac troponin, provides connectivity to *in vivo* results. These 3-dimensional structures provide a multicellular environment to more accurately reflect the complexity required when investigating the mechanism of action or the toxicity of the compounds. In conclusion, hES-CMC UniSpheres™ represent the convergence of authentic human cells with a robust, stable and reproducible format designed for extended in vitro phenotypic assays.

Poster Board Number: F-2015

INTERACTION OF PLURIPOTENT AND MULTIPOTENT STEM CELLS AND CARDIOMYOCYTES WITH HUMAN CARDIAC EXTRACELLULAR MATRIX

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Background: Stem cell therapies for heart patients are a great hope in regenerative medicine, and first clinical trials already started in the 1990s. Upon delivery of cells into the patient heart, the surrounding extracellular matrix (ECM) may influence their behavior and thereby the regenerative potential of the cells. Our goal is to study the interaction between human cardiac ECM and stem cells or cardiomyocytes *in vitro*. For this purpose, we used left ventricular human heart tissue, which we decellularized in order to obtain cardiac ECM by removing cardiac cells. After decellularization, we recellularized the matrix with different types of stem cells and cardiomyocytes and studied cell reactions upon seeding in the matrix. Methods: ECM was obtained by decellularization of human left ventricular heart tissue in lysis solution, sodium dodecyl sulphate (SDS) and serum. Cell types used for recellularization were human cord-blood mesenchymal stem cells (cb-MSCs), murine embryonic stem cells (mESCs), murine induced pluripotent stem cells (mIPSCs), cardiomyocytes derived from these cells (iCMs) and HL-1 cells (=murine cardiomyocyte tumor cell line). Crosstalk between extracellular matrix and stem cells was studied by viability assays (MTS assay, Promega), apoptose assays (Caspase 3/7 Glo assay, Promega), histology and immunohistochemistry. Results: All cell types attached to the matrix and were viable on the scaffold for the test period of 20-21 days. Injection of cells with a syringe needle did not improve attachment compared to pipetting them on top of the matrix. The viability of cb-MSCs declined on the matrix, whereas control cells seeded in a well showed increasing or at least stable viability. By contrast, mESCs and miPSCs showed a higher increase of viability on the matrix compared to control cells. Although only a proportion of the seeded cells attach to the matrix, viability of the matrix cells outdistanced that of control cells after several days of culture. One experiment included recellularization with a mix of murine bone-marrow MSCs and iCMs. Viability of the mixed cell population was higher than each cell type alone, suggesting a positive effect of cardiomyocytes on MSC proliferation. Next to studying viability, HL-1 and iCM cells were also subjected to apoptosis assays. Within the first three days after recellularization, these cells consistently showed lower Caspase 3/7 activation in wells with matrix compared to wells without matrix. Conclusion: Human heart ECM, obtained by SDS/serum-mediated decellularization, supported the proliferation of miPSCs and mESCs. In the case of MSCs, a mixed cell population including cardiomyocytes showed higher proliferation than MSCs on matrix alone. The findings may help to explain the failure of clinical cell therapies with MSCs in the cardiac field, but they also provide hope that MSCs may have benefits as supporting cells for cardiomyocyte transplantation.

Poster Board Number: F-2016

APPROPRIATE METHODS FOR ISOLATION OF HUMAN CARDIAC PRECURSOR CELLS FOR CLINICAL USES

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In recent years, isolation of multi-potent cardiac stem cells (CSCs) from mammalian hearts and their confirmed application for heart regeneration have attracted researchers' attention to this new stem cell source as a heart regenerative tool. Therefore, finding the best methods to isolate these cells and also getting optimum number of cells for transplantation are as important issues. In this study, isolation of CSCs from patient hearts' biopsies with congenital diseases was done with three different protocols based on their different properties: migration from cultured explants, clonogenic potential and expression of stemness markers i.e. c-kit. After characterization of isolated cells, changes in transcriptom of cells at different passages were analyzed using microarray. Isolated cells were positive for mesenchymal stem cells (MSCs) markers and negative for hematopoietic lineage markers. They expressed cardiac-specific transcription factors except Nkx2.5 but didn't express cardiac muscle structural genes. They showed myogenic potential after differentiation. Microarray data showed significant changes at mRNA level after subsequent passages (between passages 9-12) ($p \leq 0.05$). Our study showed that isolated cells were almost similar

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in their properties and differentiation potential but differed in the cells numbers after a definite passage; so it is possible to use one of these methods only based on the sample size and the desirable final cells count.

Poster Board Number: F-2017

GENERATION OF RAINBOW REPORTER LINES IN HUMAN PLURIPOTENT STEM CELLS FOR CARDIAC SUBTYPE SPECIFICATION

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Pluripotent human embryonic stem cells (hESC) have the potential to differentiate to any cell type of the human body. This characteristic has sparked researchers to study the use of hESC for regenerative medicine, drug screenings and embryonic development. We have recently optimized differentiation of hESC to cardiomyocytes, including growth factor directed differentiations as monolayers or as three-dimensional aggregates (embryoid bodies or EBs). Previously, we have demonstrated that hESC-derived cardiomyocytes (hESC-CM) faithfully recapitulate the early molecular events during embryonic development. Recently, we have generated a cardiac reporter line by introducing Green Fluorescent Protein (GFP), in the genomic locus of the early cardiac transcription factor NKX2-5, which enables us to visualize the derivation of NKX2-5+ cardiomyocytes during *in vitro* differentiation and purify these cells by Fluorescent Activated Cell Sorting (FACS). Transcription factor Mesp1 is described as key regulator of pre-cardiac mesoderm and represents the earliest marker of cardiovascular progenitors. In order to identify molecular mechanisms underlying early myocardial differentiation during hESC-CM differentiation *in vitro*, we performed Mesp1 gene targeting in hESC, leading to fluorescent mCherry expression under control of the Mesp1 promoter. This Mesp1-mCherry hESC line will also be generated in the NKX2-5-GFP hESC line, Fluorescent Mesp1 expression, enables us to visualize and purify hESC-derived cardiac mesodermal progenitors. The combination of different transcription factor-coupled fluorescent reporters in this so-called "rainbow" hESC cell line, covering sequential stages of the cardiac lineage, will allow us to identify and characterize pathways for specific subtypes of the cardiac lineage at early and later stages during differentiation. Furthermore, the genetic tools can also be applied to human induced pluripotent stem cells. This knowledge will be further used to direct the subtype differentiation process into a desired cardiac cell type, which then may be used for developmentally related topics and clinical and/or pharmaceutical applications.

Poster Board Number: F-2018

TRANSITION IN CARDIOGENIC TO ANGIOGENIC POTENTIAL OF HUMAN CARDIAC PROGENITOR CELLS OCCURS WITH AGE

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Background: Human cardiomyocyte turnover occurs even after birth, but rarely happens later in life. And cardiac progenitor cells take part in cardiomyocyte turnover. So we examined whether human cardiac progenitor cells become senescent and change their roles with age. Methods: Human cardiac progenitor cells were obtained from discarded myocardial tissue at cardiac surgery between the ages of 0 and 6. Each of cardiac progenitor cells was classified into two groups, neonates and infants, according to the age at surgery. Cell proliferation activity, telomere length, telomerase activity, and senescence associated beta-gal staining were examined to see age-dependent differences between the groups. Moreover, cardiac differentiation potential by coculture with neonatal rat ventricular myocytes and angiogenic potential by tube formation assay were examined. Results: Cardiac progenitor cells from neonates showed higher proliferative capacity, longer telomere length and less senescence associated beta-gal activity than those from infants, whereas level of telomerase activity did not change between in both groups. Because proliferation capacity could not account for their phenotypes, we examined differentiation potential of cardiac progenitor cells with age. Cardiac progenitor cells from neonates differentiate into more cardiomyocytes and form fewer vessels than those from infants. Conclusion: Collectively, human cardiac progenitor cells may become senescent with age *in vivo*. And transition in cardiogenic to angiogenic potential of human cardiac progenitor cells may be one of crucial mechanisms of cardiomyocyte turn over with age.

Poster Board Number: F-2019

SSEA-4+ CD34- CELLS IN THE HUMAN ADULT HEART MAY REPRESENT A PROGENITOR POPULATION WITH CARDIOMYOGENIC DIFFERENTIATION POTENTIAL

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Introduction: The stage-specific embryonic antigens (SSEAs) were originally discovered in the late 70's, and have been used to describe differentiation state of embryonic stem cells (ES cells). In human ES cells, SSEA-3 and 4 are highly expressed in undifferentiated cells, while expression of SSEA-1 is low. Upon differentiation, SSEA-3 and 4 are downregulated whereas SSEA-1 is upregulated. SSEA-4 has also been described as a marker for adult stem cells, such as mesenchymal stem cells and very small embryonic like cells (VSEL)

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in the bone marrow. Cells expressing SSEA-4 have also been identified in human neonatal cardiac tissue. However, there is currently no data on the expression of SSEAs in human adult cardiac tissue. Purpose: To investigate whether there are cell populations in the human adult heart which express SSEAs. Methods: Right atrial biopsies were obtained after informed consent from patients undergoing cardiac surgery. The tissue was dissociated and the cardiomyocyte depleted cell suspension was subjected to multi color flow cytometry. Expression of SSEA-1, 3 and 4, as well as hematopoietic marker CD45 and EPC / endothelial marker CD34 were investigated. Cells positive for stem cell markers were purified using FACS sorting and analyzed for mRNA expression. Results: Directly isolated cells contained populations of SSEA-1 (0.8% ± 0.2%), SSEA-3 (13.0% ± 5.9%) and SSEA-4 (5.2% ± 1.2%) positive cells. The SSEA-1+ population dimly expressed the hematopoietic marker CD45, while SSEA-3 and SSEA-4 positive cells were mostly CD45-. The SSEA-4+ population could be further subdivided based on CD34 co-expression. To further characterize the SSEA-4+ population, cells were FACS sorted for quantitative real-time PCR analysis. The SSEA-4+ CD34- population was found to have a very high expression of the cardiac specific genes NKX2.5 and TNNT2. No elevated mRNA expression of the cardiac stem cell associated marker C-KIT could be found in the SSEA-4+ population, regardless of CD34 expression. Conclusions: Biopsies from human right atrium contain cells expressing SSEAs. A subpopulation including SSEA-4+ CD34- CD45- cells might represent a novel cardiac progenitor population, with potential of cardiomyocyte differentiation.

Poster Board Number: F-2020

COMPLETE GENETIC CORRECTION OF CARDIOMYOCYTES FROM DMD PATIENT-DERIVED HUMAN INDUCED PLURIPOTENT STEM CELLS

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Duchenne Muscular Dystrophy (DMD)-associated cardiac diseases are emerging as a major cause of DMD patient morbidity and mortality. Cardiomyopathy is present in about 90% of DMD patients and heart failure is thought to be responsible for 20% of deaths. Many experimental therapies target skeletal muscle but they fail to improve cardiac function and therapies restricted to skeletal muscles alone in DMD patients, have been shown to accelerate heart disease. Oshimura and colleagues recently demonstrated the complete correction of human induced pluripotent stem cells (hiPSc) derived from a DMD patient, using a human artificial chromosome carrying a full-lenght genomic dystrophin sequence (DYS-HAC), including the associated regulatory elements. However, aiming at an hiPSc-mediated gene therapy for DMD patients, it is necessary to differentiate the genetically-corrected hiPSc into relevant cell types. The maintenance of the DYS-HAC during hiPSc differentiation remains still an open issue, as well as the correct activation of the complex mechanism regulating dystrophin expression, including the multiple promoters activities and the exon-skipping and exon-scrambling events, that are the finely regulated in a development- and tissue-specific way. In this work we derived, for the first time to our knowledge, patient-specific, genetically-corrected human cardiomyocytes (hCMs) from DYS-HAC-containing hiPSc and we tested the restoration of different dystrophin isoforms expression. hCMs were successfully obtained from hiPSc using a procedure based on the protocol first described by Keller and colleagues. The application of this procedure resulted in the obtainment of a 20% of spontaneously-contracting embryoid bodies (EBs). Subsequent adhesion on substrates with physiological stiffness allowed the obtainment of functionally-differentiated hCMs, characterized by a remarkable sarcomeric organization and displaying calcium transients upon electrical stimulation, typical of calcium cycling during contraction. Immunofluorescence and RT-PCR analyses revealed the expression of the cardiac markers GATA4, NKX2.5, cTnT, α-actinin, MLC2A, Cnx43. DYS-HAC-mediated dystrophin expression restoration was assessed by RT-PCR analyses, using primers designed on deleted exons in the DMD patient. Gene expression analyses on EBs at different stages of the differentiation process reveal the expression of multiple dystrophin isoforms. Immunofluorescence analyses confirmed a remarkable dystrophin expression, correctly localized at membrane level, on cTnT-positive hCMs. Taken together, these results demonstrate that HAC-mediated genetic correction allow restoration of multiple dystrophin expression at cardiac level. The obtained hiPS-derived CMs can represent a valuable cell source for *in vitro* modeling of DMD cardiac disease. In addition, being genetically-corrected and patient-specific, these CMs could represent a further step towards the development of hiPSc-mediated gene therapy for DMD patients.

Poster Board Number: F-2021

DERIVATION OF FUNCTIONAL CARDIOMYOCYTES FROM HUMAN PERIPHERAL T CELL DERIVED INDUCED PLURIPOTENT STEM CELL

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[Background] Recently, induced pluripotent stem cells (iPSCs) are expected to be cell sources for genetic disease models and new revolutionary therapies. But the generation of iPSCs needs invasive approach such as skin biopsy. To solve this problem, we developed the novel generation method of human peripheral T cells derived iPSCs (TiPSCs). TiPSCs have prominent advantages for clinical application because of minimally invasive approach to obtain patients cells. But it was unknown that TiPSCs which had TCR rearrangement in its genome can differentiate into functional cardiomyocyte. To address this issue, we differentiate TiPSCs into cardiomyocyte and investigated morphology, gene expression pattern and electrophysiological properties of TiPSCs-derived cardiomyocytes. [Methods and Results] TiPSCs were differentiated into cardiomyocytes by floating culture. RT-PCR analysis and Immunohistochemistry showed that TiPSCs-derived cardiomyocytes properly expressed cardiomyocyte markers and ion channels, and showed healthy cardiomyocyte morphologies. Multiple electrode arrays were used for characterization of TiPSCs-derived cardiomyocytes. The application of ion channel inhibitors revealed that TiPSCs-derived cardiomyocytes have normal electrophysiological responses in terms of beating rate and the field potential waveform. [Conclusions] We successfully differentiated TiPSCs into cardiomyocytes and TiPSCs-derived cardiomyocytes showed functional cardiomyocyte properties in morphology, gene expression pattern and typical electrophysiological features which respond to several drugs. These TiPSCs-derived cardiomyocytes can become disease models for understanding the mechanisms of cardiac disease and lead to new revolutionary therapies.

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BMI1 MOUSE CARDIAC STEM CELLS CONTRIBUTE TO THE HOMEOSTASIS OF ADULT MAMMALIAN HEART WITHOUT INJURY MEDIATION.

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Adult mammalian heart has been long considered a terminally differentiated organ, unable to replace cardiomyocytes. But the paradigm of a post-mitotic heart has been challenged during the last years. Compelling evidences suggest that the mammalian heart is in continuous, but low, turnover and possesses an intrinsic regenerative potential: new cardiomyocytes are formed during the adulthood. Among all the contenders that could be involved in this process, CSCs may be leading a central role. The adult murine heart appears to contain a reservoir of resident stem cell population (cardiac stem cells: CSCs) defined by the expression of different stem-cell surface markers. Nonetheless, these populations may be related, and even, partially over-lapping. Reliable markers are need to shed light: Bmi1 has been established as a new nuclear marker to identify and define adult stem cell compartments in some tissues, being a master regulator of the self-renewal of hematopoietic and neural systems among others. The main gap that has followed CSCs field has been the absence of a lineage tracing analysis that let us know the localization of adult CSCs and trace their progeny. We have established, for the first time, a lineage tracing strategy taking advantage of the Bmi1IresCreER mice model. The adult mouse heart contains a resident stem cell population associated by the expression of Bmi1 (B-CSCs). B-CSCs constitute a subpopulation of the Sca-1+ fraction and are negative for the expression of c-Kit and the hematopoietic marker CD45. *In vivo*, Bmi1+ CSCs are detected at low levels, localized in very cramped cell structures (niches) and associated with coronary vessels. We have traced the progeny of these cells through the three main lineages of the heart: endothelium, smooth muscle and cardiomyocytes, and for the fist time, the results indicate the contribution of adult endogenous CSCs into the homeostasis of adult heart without any injury mediation.

Poster Board Number: F-2023

PROARRHYTHMOGENIC AND REPOLARIZATION RISK STRATIFICATION USING VIRAL FREE HUMAN INDUCED PLURIPOTENT STEM CELL DERIVED CARDIOMYOCYTES

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Proarrhythmogenic risk and altered repolarization of cardiac cells by pharmaceuticals is an important determinant for safety evaluations. We recently generated and demonstrated that viral free induced pluripotent stem cell derived cardiomyocytes (hiPSC-CMs) form a functional electrotonic syncytium, which could provide an effective *in vitro* model for early screening for cardiotoxicity of pharmaceutical agents. Aim: We evaluated the effects of anti-arrhythmic drugs (Class I-IV) on hiPSC-CMs in an attempt to study their ability for evaluating repolarization and drug induced proarrhythmias *in vitro*. Methods: Microelectrode arrays (MEA) were utilized to record extracellular field potentials (FPs) as well as effects of several anti-arrhythmic drugs. Results: Our results demonstrated that sotalol, amiodarone, quinidine and flecainide all caused a significant prolongation of repolarization phase (FP durations, cFPDs) in a dose dependent manner. There was about 1.5-2.5 fold increase in FP durations depending on the drug under evaluation. While Class I and III prolonged cFPDs, Class IV reduced cFPDs whereas Class II had no significant effects on cFPD. Non-linear dose dependent (ranging 10-9-10-4 M) regression analysis utilizing one phase decay algorithms clearly indicated a direct relationship between cFPD prolongation doses and human estimated unbound therapeutic plasma concentration (ETPC unbound) range for each drug, except amiodarone. In accordance to their expected clinical reactions, hiPSC-CMs treated with higher doses (10-100 μM) of these drugs induced arrhythmias indicating the ability of hiPSC-CMs to detect proarrhythmogenic potential of drugs. While quinidine treatment induced ventricular tachycardia-like arrhythmia, flecainide provoked bi- or trigeminy-like arrhythmias. Rhythm disturbance was accompanied by 2-3 folds increase in beating frequencies of these iPSC-CMs as expected in clinical scenario. Furthermore, class IV drugs demonstrated cessation of contractions whereas Class II had decreased beating frequencies. Conclusion: Our results for the first time demonstrate that hiPSC-CMs could be effectively utilized for early evaluations of drug induced proarrhythmic effects as well as cardiac membrane repolarization *in vitro*, as an effective tool for early drug screening. These results coupled with the ability to generate patient specific cardiomyocytes will be a valuable human *in vitro* model for testing experimental drugs.

Poster Board Number: F-2024

WNT16 MODIFICATION OF CARDIAC STEM CELLS BOOSTS REPAIR CAPACITY IN ISCHEMIC REPERFUSED (IR) HEART

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The Wnt gene family promotes developmental cardiogenesis so we examined the role of Wnt16 in CSC differentiation, angiogenesis and cell migration, as well as the reparative and regenerative effect of Wnt16-expressing CSC in IR rat heart. Methods: Rat Wnt16-GFP-CSC were generated and transfection efficiency confirmed using immuno-blot and fluorescence. Rat hearts (grouped into media control, unmodified CSC and Wnt16-GFP-CSC treatments; n=3 each) were subjected to 30min ischemia followed by reperfusion and injection of ~1x106 cells or media into infarct-border zone (BZ) at intervals of 2mm. Heart tissue was harvested at 48h and 4w to examine cytokine levels, blood vessel density, and ischemic area/infarct size. Results: Wnt16-GFP-CSC showed positive expression and led to reduced ischemic area and infarct size compared to control at 48h (ischemic area 30.8±1.4%, infarct 17.9±1.9% of LV vs 50.8±2.7%, 37.9±3.9%, $p<0.05$). At 4w LV fibrotic area was 20.4±1.35% with Wnt16-GFP-CSC, 35.9±1.8% with unmodified CSC and 52.3±2.1% in control ($p<0.05$). There was increased 4w expression of angiogenic VEGF and Flk-1, and anti-apoptotic factors phosphorylated-GSK3β and Bcl-2. At 4w cytokine levels (MIP1α, RANTES, L-selectin) had decreased compared to control and blood vessel formation increased in response to Wnt16-GFP-CSC treatment in BZ by 50.41±2.52%. Conclusion: CSC pre-conditioning with Wnt16 enhances their reparative/regenerative effect in IR rat heart with resulting decrease in infarct size both at 48h and 4w, while increasing angiogenic and anti-apoptotic factors and creating favourable changes in cytokine profile at 4w.

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REACTIVE OXYGEN SPECIES PROBABLY PLAY DUAL ROLES IN GENOMIC STABILITY OF STEM CELLS

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Background: Chromosomal abnormalities are frequently found in human embryonic and adult stem cells after long-term culture, but the precise mechanisms are still unclear. Stem cells are generally cultured in media equilibrated with 95% air and 5% CO2 (~20% O2), which is much higher than in the *in vivo* physiological microenvironment (about 1-5% O2, depending on the tissue). As oxidative stress, an increased production/accumulation of reactive oxygen species (ROS) in cells, is well-known to induce DNA damage, we tried to reduce the incidence of genomic alterations by reducing oxidative stress. Methods and Results: Human cardiac stem cells were cultured under physiological oxygen (5% O2) or by adding antioxidants to routine culture media. Culture of cardiac stem cells in 5% O2 moderately decreased the intracellular level of ROS and reduced the incidence of chromosomal abnormalities, but the addition of antioxidants to the medium increased chromosomal abnormalities of cells with a dramatic dose-dependent decrease of ROS. To identify whether an excessive decrease of ROS induces DNA damage, we quantified γ-H2AX foci in stem cells as a function of antioxidant concentration. The effects were biphasic: the percentage of stem cells with γ-H2AX foci was decreased at low antioxidant concentrations, but increased at higher doses. Interestingly, the number of γ-H2AX foci was minimal at modest concentrations of antioxidants, the same concentrations that drive ROS to "physiological" levels. Furthermore, we found that excessive suppression of ROS by high concentrations of antioxidants down-regulated ATM, ATR, and other DNA repair factors, thereby favoring genomic instability. Conclusion: Our data suggested that ROS probably plays dual roles in inducing genomic instability of stem cells. Namely, ROS are likely required to activate the DNA repair pathways for maintaining genomic stability in stem cells, but high ROS (oxidative stress) is also well known to induce DNA damage.

Poster Board Number: F-2026

NUMB ACTIVITY IS REQUIRED FOR CARDIAC CELL LINEAGE AND FATE DETERMINATION

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During cardiac development, precardiac cells must make appropriate fate decisions within a complex and dynamic environment. However, the pathways and mechanism regulating the specification and fate of cardiac progenitors are yet to be determined. Numb is an evolutionarily conserved protein with important roles in asymmetric cell divisions during animal development. We found that extinguishing expression of Numb in cardiovascular progenitors resulted in failure of their specification with severe defects in heart and vascular morphogenesis, causing an early embryonic lethality. Consistently, inhibition of Numb activity in embryonic stem cell-derived cardiac progenitors impaired their differentiation into cardiac cell lineages in-vitro. We have established a critical novel role of Numb in the specification of cardiac progenitors and cardiac development.

Poster Board Number: F-2027

MITOCHONDRIAL DNA DELETIONS IN ADULT MOUSE CARDIAC SIDE POPULATION CELLS

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We investigated the presence and potential role of mitochondrial DNA (mtDNA) deletion mutations in adult cardiac stem cells. Cardiac side population (SP) cells were isolated from 8- and 12-week-old mice and standard polymerase chain reaction (PCR) was used to screen for the presence of mtDNA deletion mutations in freshly isolated and cells cultured to passage 10. When present the abundance of mtDNA deletions was analyzed in single cell colonies. The effect of different levels of deletion mutations on SP cell growth and differentiation was determined. MtDNA deletion mutations were found in freshly isolated and cultured cells from both 8- and 12-week-old mice. While there was no significant difference in the number of single cell colonies with mtDNA deletions from any of the above groups, the abundance of mtDNA deletions was significantly higher in the cultured cells, as determined by quantitative PCR. We also found that cells harboring high levels of mtDNA deletions (i.e. deleted mtDNA comprised more than 60% of total mtDNA) also showed slower proliferation rates and decreased differentiation capacities. MtDNA deletion mutations could be used as markers to assess adult stem cells that exhibit stability over extended periods of culture.

Poster Board Number: F-2028

BROWN ADIPOSE TISSUE DERIVED-CELLS DIFFERENTIATE INTO CARDIAC CONDUCTION AND PACE-MAKER CELLS IN VITRO AND IMPROVE COMPLETE AV BLOCK *IN VIVO*

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An adipose tissue is one of the sources of mesenchymal stem cells, which have the potential to differentiate into various types of cells. Here we report that adipose tissue-derived mesenchymal stem cells differentiate into cardiac conduction system and pacemaker-like cells. Brown adipose tissue (BAT) were isolated from interscapular area of mice and enzymatically digested before culture. The cell surface marker analysis of isolated BAT-derived cells showed CD29 100%, CD31 17%, and CD45 6.4%. Round or tube-like cells in some of the colonies showed spontaneous beating at 4-7 days after culturing of BAT-derived cells. RT-PCR analyses revealed that BAT-derived cells expressed Nkx2.5, GATA6, Tbx5, ANF, MEF2C, cardiac alpha actin and MyoD. Immunocytochemical analysis revealed that the most of round and tube-like cells in beating colonies expressed sarcomeric alpha actinin (SA), alpha myosin heavy chain, beta myosin heavy chain, and cardiac Troponin T, cardiac Troponin I. Some of the tube-like cells showed fine sarcomeric structures. Patch-clamp techniques revealed that spontaneous electrical activity appeared between -40 and -60 mV. The shape of action potential from beating round and tube like cells showed properties of both cardiac pacemaker and neuronal cells. Next, we assessed the ability of the BAT-derived cells to adequately respond to adrenergic and muscarinic stimuli, we evaluated the possible chronotropic changes induced by administration of the β-agonist isoproterenol and the muscarinic agonist carbamylcholine . Isoproterenol administration resulted increase in the spontaneous beating frequency. In contrast, carbamylcholine administration resulted in decrease in

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the spontaneous beating frequency. BAT-derived cells expressed several cardiac conduction system and pace-maker cell marker genes, such as Tbx5, HF-1b, connexin 40, connexin 45, MinK, and HCN1 to 4, which are necessary for pace-maker activity. Real-time PCR studies showing that pace-maker cell marker genes, such as Tbx3, Tbx18, and Kir2.1 was increased. To examine the regenerative potential, we created complete atrioventricular(AV) block in mice and injected GFP positive primary beating BAT-derived cells or passaged white adipose tissue (WAT) derived cells which did not show any cardiac or skeletal characters, intramyocardially into around the AV node. At 1weeks after transplantation, 50% of BAT-derived cells injected mice improved sinus rhythm or 2:1 AV block(n=8), but all WAT-derived cells injected mice continued complete AV block(n=4). Immunocytochemical analysis revealed that GFP and SA positive cells exist in atriam and ventricular septum in BAT-derived cells injected heart. And some GFP positive cells co-expressed several cardiac conduction system and pace-maker cell marker genes. These findings suggest that BAT-derived cells differentiate into cardiac conduction system and pacemaker-like cells in vitro and *in vivo*, and may become a useful cell source for arrhythmia therapy.

Poster Board Number: F-2029

CALRETICULIN DEFICIENT ($CRT^{-/-}$) MOUSE EMBRYONIC STEM CELL MODEL ELUCIDATES THE CALCIUM-DEPENDENT NUCLEAR PORE COMPETENCY REQUIRED FOR CARDIAC DEVELOPMENT

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Calreticulin deficiency results in myocardial developmental defects culminating in embryonic lethal heart failure. Recent studies have linked the loss of this calcium-binding chaperone to a failure in myofibrillogenesis. Here we used the mouse embryonic stem cell model of calreticulin deficiency ($crt^{-/-}$) to dissect the molecular basis of calreticulin-mediated dysregulation during cardiac development. $crt^{-/-}$ deficiency was found to have disrupted nuclear import of the cardiac transcription factor MEF2C, and diminished nucleocytoplasmic transport of nuclear localization signal-containing peptides as well as histone H1, a constitutive nuclear protein. Expression profiling of $crt^{-/-}$ derived cardiomyocytes highlighted impact on the nuclear pore complex with significant downregulation in nuclear transport machinery, including nucleoporins and cargo transport proteins. Alteration in protein content resulted in a conformation change of the $crt^{-/-}$ nuclear pore architecture as demonstrated by a decreased diameter and diminished probability of conduit occupancy when compared to wild type stem cell-derived cardiomyocytes at nanoscale resolution. The defect in nuclear pore complex function was associated with abnormal myofibrillogenesis. Correction of reduced intracellular calcium pools, seen with calreticulin deletion, reinstated nuclear pore microarchitecture and rescued nuclear import resulting in normalization of myofibrillogenesis. Thus, calreticulin-dependent calcium modulation is essential for maintenance nuclear pore transport competency during myofibrillogenesis, identifying a new role for calreticulin as a critical regulator of nucleocytoplasmic communication during cardiac differentiation.

Poster Board Number: F-2030

METABOLIC SELECTION FOR PLURIPOTENT STEM CELL DERIVED CARDIOMYOCYTES

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Heart-regenerative cell therapy using pluripotent stem cells (PSCs) including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) is a promising divergent therapeutic strategy for patients with severe heart failure. Mass production of highly purified cardiomyocytes is a critical bottleneck in realizing heart regenerative therapy. Our recently established non-genetic purification method of cardiomyocytes using mitochondrial dye is efficient but not suitable to produce clinical-scale cardiomyocytes due to the usage of FACS. This study was designed to establish a large-scale purification method for PSC-derived cardiomyocytes based on cell-specific differences in metabolism and nutrition source. We first approached this issue by focusing on the possible metabolic differences between cardiomyocytes and ESCs by transcriptome and metabolome analyses. Transcriptome analysis delineated marked differences in isozyme expression and mRNA levels between metabolic pathways including glycolysis and the TCA cycle. Fluxome analysis using [^{13}C]-labeled glucose revealed that ESCs used glucose mainly for biomass synthesis, while cardiomyocytes used glucose primarily for ATP production via TCA cycle. Lactate was discarded by ESC, but it was incorporated into cardiomyocytes. Taken together, we developed a novel method for purifying the bulk of PSC-derived cardiomyocytes using original culture media. This culture media enabled us to obtain > 98.5% pure cardiomyocytes derived from human ESCs which did not form teratoma when transplanted into immunodeficient mice testes. We investigated the molecular mechanism to determine why cardiomyocytes can survive for the long period in the lactate-supplemented and glucose-starved medium by fluxome analysis using [^{13}C]-labeled lactate. Surprisingly, they can produce some biomass from lactate via gluconeogenic pathway. In this study, we report a novel method to purify a bulk of cardiomyocytes from PSC-derivatives based on the findings from transcriptome and fluxome analyses. We believe that our novel and inexpensive method termed "Lactate Method" will resolve the bottleneck and directly facilitate human heart-regenerative therapies.

Poster Board Number: F-2031

DIFFERENTIATED CELL-SPECIFIC GENE BODY DNA HYPOMETHYLATION CONTRIBUTES TO TRANSCRIPTIONAL EFFICIENCY IN CELL TYPE-SPECIFIC EXPRESSION PATTERNS.

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[INTRODUCTION] Efficient transcription of tissue-specific genes is important for the maintenance of functionality of differentiated cells. In mammals, non-promoter regions are generally highly methylated, resulting in global repression of gene transcription. This suggests that a reduction of gene body DNA methylation in certain genes would enhance transcription of these genes. Escape from gene body methylation could be a cell type-specific strategy to allow efficient transcription in a select set of genes. In this study,

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we challenged to address whether the alteration of DNA methylation distribution may contribute to the cell type-specific gene expression patterns. [METHODS AND RESULTS] We analyzed the genome-wide patterns of transcriptional and DNA methylation status of embryonic stem (ES) cells and purified mouse cardiomyocytes from several developmental stages. A massively parallel sequencing-based high-throughput analytical technique for DNA methylation, HELP-tagging method, was used for the analysis of global epigenetic status. In comparison with their gene expression patterns, the gene body DNA methylation levels of multiple genes showed a negative correlation with gene expression in cardiomyocytes compared to ES cells. In contrast to global DNA methylation levels, the decline in DNA methylation levels of cardiac genes, for example in the entire myosin heavy chain locus, was induced before birth and was subsequently maintained. Overall, the hypomethylation was more enhanced in the promoter-proximal half of the gene body, suggesting the possibility of transcription-associated escape of DNA methylation. [CONCLUSION] We speculated that gene body DNA hypomethylation of the cell type-specific gene subpopulation might be actively induced during cardiomyocyte differentiation, in contrast to the global DNA hypermethylation in pluripotent stem cells. Since the negative correlation of gene body DNA methylation levels and gene transcription was not observed in ES cells, we propose that the gene body DNA hypomethylation of cell type-specific gene loci is a strategy for the efficient transcription of a set of genes in differentiated cells.

Poster Board Number: F-2032

INTERPLAY BETWEEN P63 ISOFORMS, ACTIVIN-A AND IGFBP7 DURING CARDIOGENESIS OF EMBRYONIC STEM CELLS

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p63 is a transcription factor, member of the p53 family, which plays a pivotal role in epidermal development. p63 gene produces two main isoforms, TAp63 and ΔNp63, which have distinct gene expression profile and opposite functions on epithelial homeostasis and cancer. While TAp63 isoform can promote apoptosis, the ΔNp63 isoform plays a role in cell proliferation, cell adhesion and epidermal development. We have recently discovered an unexpected role of p63 in heart development and mammalian cardiogenesis. We detected that p63-null embryos suffer from severe congenital cardiopathy. *In situ* hybridization revealed that p63 is produced by embryonic visceral endoderm at early stage. By the use of murine embryonic stem cell (mES) model, which recapitulates successive steps of early cardiogenesis, we demonstrated that TAp63 controls, in a cell-nonautonomous manner, cardiac progenitor differentiation. Inhibition of TAp63 during differentiation did not interfere with the commitment of mesodermal progenitors but prevented their commitment to cardiac progenitors and mature cardiomyocytes. In the present study, we observed that, while inhibition of TAp63 reduced cardiomyocyte differentiation, repression of ΔNp63 enhanced it. We found that this opposite regulation occurred through the direct modulation of the expression of Activin-A gene, a member of the TGFβ superfamily. This modulation demonstrated by both Activin-A promoter/reporter assays and ChIP-qPCR analysis. Treatment of ES cells by SB431542, an inhibitor of Activin/Nodal pathway, abolished the repressive effect of ΔNp63 on cardiogenesis. IGFBP7, a member of soluble secreted proteins that bind insulin-like growth factors, has been suggested to bind Activin-A. Moreover, a recent report of a germ line mutation in IGFBP7 gene in humans suggests a specific developmental role in the cardiovascular system. Finally, our recent transcriptome analysis of laser captured-embryonic p63-null skin revealed that IGFBP7 is a target gene of ΔNp63. Accordingly, we found that during mES-driven cardiogenesis, IGFBP7 expression was enhanced by siΔNp63 and repressed by siTAp63. Furthermore, ΔNp63 and TAp63 directly regulated IGFBP7 in a competitive and opposing fashion, as repressor and activator of IGFBP7, respectively. These effects were partially abolished by SB431542 treatment, suggesting a dual direct and indirect regulation of IGFBP7 by the p63/Activin pathway. Interestingly, IGFBP7 levels affected ΔNp63 and TAp63 expression, thus creating a regulatory feedback loop. Because of their potential as cellular source for cardiac cell therapy, we tested whether similar regulation could occur in human ES cells. Remarkably, inhibition of ΔNp63 strongly enhanced cardiomyocyte differentiation of huES cells as shown by the significant enhancement of beating cells and elevated Activin-A and IGFBP7 gene expression. In conclusion, our results demonstrate the antagonistic regulation of the two p63 isoforms on Activin-A/IGFBP7 expression in a dual regulatory manner. These findings emphasize the importance of p63 in mammalian cardiogenesis.

Poster Board Number: F-2033

C-KIT POSITIVE CARDIAC STEM CELLS FROM APPENDIX OF THE RIGHT ATRIUM AND LEFT VENTRICULAR ANEURYSM POSSESS DIFFERENT PROPERTIES

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Accumulating evidence indicates that the adult mammalian myocardium, including that in humans, contains a small pool of tissue-specific progenitor cells that can replenish the cardiomyocyte and vascular cells population and represent new development in stem cell-based cardiac regenerative medicine. The aim of the present study is to characterize properties of c-kit+ cardiac stem cells in the human atrial heart tissue (appendix of the right atrium (ARA)) and aneurysm, harvested during surgical operations from patients with coronary artery disease. Using immunohistochemistry and flow cytometry we have found that c-kit positive cells in the ARA and aneurysm tissue expressed stem cell markers (MDR1, c-met, Igf1R, N-cadherin) and characterized by the absence of hematopoietic markers (CD34, CD45). Part of c-kit positive cells in the ARA proliferate (express Ki67) and differentiate to cardiomyocytes and smooth muscle cells (express Gata4, sarcomeric actinin, smooth muscle actin). C-kit positive cells in the aneurysm tissue did not proliferate, did not express markers of cardiomyocytes and vascular cells and expressed cell cycle inhibitor p21Cip/Waf1. Number of c-kit+ cells in the ARA were higher in women younger than 60 years compared to older women and men below and up 60. It is positively correlated with the severity of coronary artery atherosclerosis and anterior-posterior size of the left atrium, but do not dependent on the presence of risk factors for cardiovascular disease (hypertension, diabetes mellitus, hyperlipidemia, obesity, smoking). Using explant culture following by magnetic cell sorting c-kit positive cells could be successfully isolated from human heart tissue and expanded *in vitro*. C-kit positive cells from ARA had a more pronounced

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proliferative capacity, than c-kit cells of the aneurysm. C-kit+ cells from ARA were clonogenic and expressed genes of pluripotency (Oct4, Sox2, Klf4, C-myc, Nanog). Cultured c-kit positive cells from ARA had ability to form cardiomyocyte-like and endothelial-like cells after cultivation in differentiation mediums. They were capable to form endothelial-like structures *in vitro* and secreted angiogenic growth factors (VEGF and HGF). C-kit positive cells from aneurysm tissue had a limited differentiation potential and did not differentiate to endothelial-like cells. Thus ARA and aneurysm tissue contain pool of c-kit+ cells that have properties of stem cells and can be isolated and expanded *in vitro*. Both tissues represent a promising source of autologous cardiac stem cells for cardiomyoplasty. However, a limited differentiation potential and proliferative capacity of cells from aneurysm should be taken into consideration.

Poster Board Number: F-2034

ANALYSIS OF DIFFERENTIATION CAPACITY TOWARD CARDIOMYOCYTES FROM PLURIPOTENT STEM CELLS

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Mouse and human somatic cells could be reprogrammed to induced pluripotent stem cells (iPS cells), which maintain an undifferentiated state similar to that of embryonic stem cells (ES cells) by transduction of four transcription factors, Oct 3/4, Sox2, Klf4, and c-Myc (4F). iPS cells can be reprogrammed from many types of cells, including fibroblasts, keratinocytes, and blood cells, such as T lymphocytes. Various methods to deliver the reprogramming factors, including retrovirus, lentivirus, sendaivirus, plasmids, and modified RNAs are reported to successfully reprogram the somatic cells into iPS cells. The embryonic stem cells are known to have marked difference in differentiation propensity between cell lines. We investigate the differentiation capacity of human iPS cells toward cardiomyocytes in in-vitro directed differentiation, and cardiomyocytes were successfully generated from human iPS cells efficiently. These derived cardiomyocytes showed electrophysiological activities similar to cardiomyocytes from normal tissues. We compared the *in vitro* differentiation propensity into cardiomyocytes in several ES/iPS cell lines, and found that several lines can produce cardiomyocytes effectively and other cell lines produce cardiomyocytes less effectively. We are analyzing the expression profiles and epigenetic status of these iPS cell lines. Our data suggest that finding the optimal cell lines to produce cardiomyocytes effectively is useful for application of the derived cardiomyocytes from iPS cells.

Poster Board Number: F-2035

TISSUE RESIDENT PDGFRA SCA1 POSITIVE MESENCHYMAL PROGENITORS WITHIN THE MURINE HEART REGULATE THE FORMATION OF CARDIAC FIBROSIS

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The cellular definition of a cardiac fibroblast has been expanded in recent years to describe a heterogeneous population of mesenchymal cells within the heart which are important mediators of repair following injury. The role of recently described cardiac-resident MSC-like cells, termed colony-forming units - fibroblasts (CFU-Fs, identified as CD45-, CD31-, Sca1+, and PDGFRa+) in the modest regenerative response of the injured myocardium has yet to be investigated. Further, in the absence of a robust regenerative response, cardiac CFU-Fs are hypothesized to be the principle cell type involved in the formation of a fibrosis within the heart. Elucidation of the cellular and molecular mechanisms regulating cardiac CFU-F's role in the axis of regeneration versus repair following myocardial injury will lead to novel therapeutic avenues to modulate these processes. Following enzymatic digestion of adult PDGFRa-EGFP murine hearts, PDGFRa+ Sca1+ were isolated and analyzed for expression of genes associated with regulation of the extra-cellular matrix (ECM). Sca1+ cells were found to express significantly more Col1a1, Col1a3, CTGF, TGFB1, Vimentin, MMP2 and MMP9 in relation to all other cell populations within the heart (PDGFRa+ Sca1-, PDGFRa- Sca1-, CD45+, CD31+) highlighting their role in the regulation of matrix proteins within the heart. The expression of ECM-related genes was further increased following daily administration of the beta-agonist isoproterenol, which also led to selective proliferation (assessed by EdU incorporation) of PDGFRa+ cells co-localizing with collagen-1+ in small infarct-like areas. Treatment with imatinib mesylate, which non-selectively inhibits PDGFRa, normalized expression of ECM-related genes, reduced proliferation and reduced the quantity of immunostained collagen-1+ depositions within the heart. Involvement of cardiac CFU-Fs in the formation of cardiac fibrosis was further evidenced by a significant increase in PDGFRa+ Sca1+ cells within the hearts of aged (1 year) MDX mice, in which a mutation in the dystrophin gene leads to cardiomyopathy and cardiac fibrosis in aged animals. Freshly sorted PDGFR+ Sca1+ cells from aged MDX mice were also found to significantly upregulate ECM-associated genes. Isolation and clonal expansion of cardiac CFU-Fs *in vitro* confirmed their multipotency towards mesenchymal lineages (cultured in osteo-, chondro- and adipogenic conditions). Treatment of cultured CFU-Fs with PDGF-AA and/or TGFB1 had potent mitogenic effects on the cultured cells, in addition to upregulating ECM-associated genes. Understanding the biological role of cardiac-resident PDGFRa+ Sca1+ mesenchymal progenitors in both physiological and pathological settings is highly relevant to the field of cardiovascular medicine. With numerous animal studies and large-scale clinical trials confirming the beneficial effects of transplantation of BM-MSCs on cardiac regeneration, it is possible that interventions targeted to manipulating cardiac CFU-Fs could lead to greater efficacy and improved clinical outcomes.

Poster Board Number: F-2036

SALL PROMOTES CARDIAC PROGENITOR CELL FATE AND FULLY CONTRIBUTE TO CARDIOMYOCYTE LINEAGES

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The heart is composed from three major mesodermal progenitor cell lineages, the first heart field (FHF) marked by Nkx2-5/Tbx5, the second heart field (SHF) marked by Islet1/Nkx2-5, and proepicardial origin (PEO) marked by WT1/Tbx18. However, why progenitor fields are located separately from common mesoderm has been unaddressed, and which indispensable factors control producing/maintaining these cardiac progenitor cells (CPCs) has been unclear. To understand these questions, we have screened out Sall genes as regulators for ectopic cardiac cell induction in the mesoderm by *in vivo*/in vitro Sall overexpression studies. Sall genes start their expression earlier than Islet1/Nkx2-5/WT1. Their expression do-

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mains are observed in anterior mesodermal layers, including major SHF that defined by Islet1, but not observed in FHF by Tbx5/Actc during embryogenesis. Sall knockout analysis resulted in severe heart defects, especially loss of OFT and RV with downregulation of progenitor expression, suggesting that Sall+ lineage lies upstream of cardiac progenitor genes and is essential for cardiogenesis. We performed lineage-tracing analysis for Sall+ cardiac progenitors, by crossing a tamoxifen inducible Sall-creERT2 mouse with a ROSA-YFP reporter mouse. Interestingly, Sall+ lineage cells substantially contributed to the embryonic heart, including OFT, RV and specific part of LV. In vitro culture system with Sall-EGFP differentiated ES cells, and sorted Sall+ cells from Sall-EGFP knock-in live mice resulted in significantly differentiated ventricular-like cardiomyocytes, SAN, smooth muscle cells. In addition, Sall-EGFP+ colonies mostly differentiated into beating cardiomyocyte (80%) with cardiac Troponin (>90%), in whereas EGFP- cells differentiated into cardiomyocytes with low efficiency (<20%). Our results investigate that Sall marks a novel factor contributing to the fully heart via two distinct (dependent-independent) pathways upon Islet1, and it's an indispensable gene for cardiac progenitor/stem cells.

Poster Board Number: F-2037

MICRORNAS REGULATE MUSCLE LINEAGE SWITCH

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MicroRNAs (miRNAs) are small non-coding RNAs regulating post-transcriptionally gene expression by degradation or translational repression of specific target mRNAs. In the 90s, lin-4 and let-7 were firstly identified as small regulatory RNAs able to control *C. elegans* larval development, by specifically targeting the 3'UTR of specific genes. These findings have introduced a novel and wide layer of complexity in the regulation of mRNA and protein expression. Lin-4 and let-7 are now considered the founding members of an abundant class of miRNAs, common in viruses, green algae, plants, flies, worms, and in mammals. In humans, the estimated number of genes encoding for miRNAs is as high as 1000 and around 30% of the protein-coding genes is post-transcriptionally controlled by miRNAs. Cardiac and skeletal muscles are powerful examples to summarize the activity of miRNAs in cell fate specification, lineage differentiation and metabolic pathways. Indeed, specific miRNAs control the number of proliferating muscle progenitors to guarantee the proper formation of the heart and muscle fibres and to assure the self-renewal of muscle progenitors during adult tissue regeneration. Conversely, other miRNAs promote the differentiation of muscle progenitors into skeletal myofibres or into cardiomyocytes. Moreover, miRNAs finely tune metabolic activity, survival and remodelling processes in response to stress, injury and chronic diseases. Recently, we showed that Sgcb-null cardiac progenitors spontaneously differentiated into skeletal myoblasts. Aberrant myogenic potential correlated with downregulation of miRNA669 family members, which directly target MyoD and prevent aberrant myogenesis. These results provide complex insights on mesodermal progenitor commitment and indicate that special tailored procedures may be required to improve cardiac stem cell-based protocols.

Poster Board Number: F-2038

DRIVING HEART PROGENITOR CELL FATE AND REGENERATION *IN VIVO* VIA CHEMICALLY MODIFIED MRNA

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A number of multipotent heart progenitors is responsible for the diversification and expansion of distinct cardiac muscle, vascular smooth muscle, and endothelial cell lineages during cardiogenesis. Unlocking the potential of these progenitors for cell-based regenerative therapeutics has been hampered by difficulties with scalability, grafting, survival, rejection, and electrical coupling. Recent studies have identified a rare number of post-natal heart epicardial progenitors, along with developmental cues that control their renewal, fate, and function during in vitro cardiogenesis in pluripotent stem cells and clonal assays. If the authentic in vivo paracrine factors could be identified and delivered in an efficient, localized, and transient manner in the injured adult heart, the in vivo cardiac delivery of paracrine factors might represent a viable alternative therapeutic strategy, akin to the known clinical utility of erythropoietin and GM-CSF to selectively augment specific blood cell lineages. In support of this concept, recent studies have suggested the presence of endogenous paracrine signals that can expand these progenitors following cardiac injury in vivo, although the precise subset of progenitors and the exact paracrine signals are largely unclear. Herein, we have identified VEGF-A as a key fate shift in the human fetal heart that expands the vascular progenitor pool in the family of multipotent human heart progenitors during ES cell cardiogenesis, and have utilized modified mRNA to transiently and efficiently express the corresponding protein at high levels in human and murine cardiomyocytes in vitro, as well as in the adult murine heart following injury. A combination of lineage tracing and FACS analysis in three independent genetically engineered mouse model systems document that VEGF-A is expanding and driving rare pre-existing WT-1 epicardial progenitors away from a previously described interstitial fibroblast like state and towards a cardiac and vascular fate following myocardial infarction. The therapeutic effect includes a 3 fold increase in WT1+ epicardial progenitors and their vascular cell lineages, an increase in capillary density, a marked reduction in cardiac fibrosis, an improvement in global cardiac function, and islands of *de novo* cardiomyocytes. In summary, modRNA is a new platform to allow the rapid in vivo assay of known or novel paracrine protein factors that can drive the expansion and fate of endogenous heart progenitors following heart injury. In addition, VEGF-A modRNA represents a novel heart progenitor cell fate shift following injury, and suggests a new cell-free therapeutic paradigm to achieve the in vivo recruitment and subsequent differentiation of endogenous heart progenitors for cardiovascular regeneration

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MODELING ARRHYTHMIAS WITH PATIENT-SPECIFIC IPS CELLS

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The recent breakthrough to generate patient-specific induced pluripotent stem cells (iPSCs) offers a new approach to model human disorders and could facilitate the development of novel therapeutic interventions. Long QT (LQT) and Brugada (Br) Syndromes (S) are genetic arrhythmogenic disorders characterized by abnormal ion channel function resulting in prolonged QT intervals or elevation in the ST segment, respectively, on an electrocardiogram. A reduction of the rapidly activating delayed rectifier potassium channel current (IKr) caused by LQTS-associated mutations in the KCNH2 gene can induce ventricular arrhythmia and cause sudden cardiac death. Here we report the development of disease-specific human iPSC lines from patients with type-2 LQTS caused by a heterozygous, 2398+1G->C splice site mutation in KCNH2. The mutation disrupts the consensus sequence of the donor splice site of intron 9 and leads to the activation of a cryptic splice site 54 bp downstream. The cryptic splicing results in a full-length KCNH2 protein with an insertion of 18 amino acids, causing a trafficking defect of the mutant channel. This was confirmed by KCNH2 immunostaining in cardiomyocytes differentiated from established LQT2-iPSC lines, thereby providing the first trafficking defect studies with patient-specific iPSC lines. Electrophysiological measurements revealed prolonged action potential-duration in KCNH2 2398+1G->C LQT2-hiPSC lines when compared to healthy control lines. Ongoing single-cell patch clamp studies should confirm that the prolongation in action-potential-duration stems from a specific decrease in IKr. In addition we will present data on iPSCs derived from a patient with two mutations in KCNH2 (355G->C and 568C->T resulting in a single amino acid substitution D119H) as well as from 2 BrS patient with a 3157G->A (E1053K) mutation. We are in the process of correcting the mutations using helper-dependent adenovirus mediated gene targeting. In addition, we are in the process of rescuing the phenotype in our splice site mutation LQT2-hiPSC lines by depleting the mutant KCHN2 mRNA using short-hairpin RNAs that target specifically the 54 bp insertion. Our work demonstrates the opportunity of hiPSC technology to study human diseases in a dish, in our case, the abnormal functional phenotype of inherited cardiac disorders.

Cancer Cells

Poster Board Number: F-2041

C-MET PROTECTS BREAST CANCER STEM CELLS FROM APOPTOSIS INDUCED BY SODIUM BUTYRATE

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Sodium Butyrate (NaBu) is regarded as a potential reagent for cancer therapy. In this study, a specific breast cancer cell population that is resistant NaBu treatment was identified. These cells possess cancer stem cell characters, such as the capability of sphere formation *in vitro* and high tumor incident rate (85%) in mouse model. Forty percent of the NaBu resistant cells express the cancer stem cells marker, the CD133, whereas only 10% intact cells present the CD133 antigen. Furthermore, the endogenous expressing c-MET contributes to the survival of cancer stem cell population from the treatment of NaBu. The CD133+ group also presents a higher level of c-MET. A combination treatment of MET siRNA and NaBu efficiently prohibited the breast cancer progression, and the incident rate of the tumor decrease to 18%. This study may help to develop a new and alternative strategy for breast cancer therapy by targeting breast cancer stem cells.

Poster Board Number: F-2042

IRRADIATED HUMAN BREAST CANCER CELLS TREATED WITH STEROID HORMONES ARE ENRICHED WITH CANCER STEM CELLS

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Exposure to ionizing radiation is known to result in an increased risk of breast cancer. There is strong evidence that steroid hormones influence radiosensitivity and breast cancer risk. Tumors may be initiated by a small subpopulation of cancer stem cells. In order to assess whether the modulation of radiation-induced breast cancer risk by steroid hormones might involve cancer stem cells, we measured by flow cytometry the proportion of cancer stem-like cells (CSCs) in irradiated T47D and MCF7 breast cancer cell lines after progesterone and estrogen treatment. We isolated a subpopulation of CSCs with high aldehyde dehydrogenase (ALDH) activity and increased ability to form tumorspheres in suspension cultures. Progesterone treatment inhibited radiation-induced cell death in both cell lines, while estrogen treatment counteracted radiation-induced cell death only in T47D cells. Exposure to ionizing radiation and estrogen treatment of non-irradiated T47D cells resulted in increased percentages of CSCs with higher tumorigenic properties. Progesterone treatment of both irradiated and non-irradiated T47D cells increased the proportion of CSCs, suggesting that progesterone might influence radiosensitivity in the breast partly by triggering the expansion of the cancer stem cell compartment.

Poster Board Number: F-2043

ENRICHED EXPRESSION OF LYMPHATIC MARKER PROTEIN, PODOPLANIN, ON LEUKEMIA STEM CELLS IN ACUTE MYELOID LEUKEMIA

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Background. Acute myeloid leukemia (AML) is organized as a cellular hierarchy initiated by self-renewing leukemia stem cells (LSCs). It is now accepted that the leukemia stem cell will not be eradicated after chemotherapy as well as hematopoietic tissues still possess both LSCs and residual normal hematopoietic stem cells (HSCs) in AML. CD34+CD38− cells can be thought of as normal HSCs as well as representative LSCs. Therefore, sophisticated LSC marker, which is distinguishable from HSCs, still remains to be investigated. Podoplanin is one of the representative markers for lymphatic endothelial cell (LEC) and use for diagnosis in tumoriogenesis as well as can function as a lymphatic endothelial progenitor cells in adult bone marrow (BM). Given previous reports raise a question whether LSCs can express lymphatic markers, but has no expression in HSCs compartment of normal blood cells including peripheral blood (PB) and BM. In this study, we first showed high expression of lymphatic marker proteins such as VEGFR-3, podoplanin in CD34+CD38− cells of AML patients and further provided a possibility of podoplanin as a promising marker for LSCs in vitro and vivo. Methods and

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Results. First, to determine expression of lymphatic marker proteins on LSCs, mononuclear cells (MNCs) from 25 AML patients and 11 healthy volunteers were isolated and subjected to qRT-PCR, FACS analysis and immunocytochemistry. Results showed that CD34+CD38− cells in AML patients highly expressed these markers, whereas, normal donor cells rarely expressed LEC markers (by FACS, leukemia condition vs normal, VEGFR-3: 24.8 ± 4.0% vs. 0.5 ± 0.2%, Podoplanin: 28.5 ± 5.8% vs. 2.1 ± 0.5%, $P < 0.05$, 1.8 to 4.8 folds by qRT-PCR) as well as AML MNCs displayed high percentage of CD34+CD38− cells compared to normal MNCs. Similarly, immunocytochemistry clearly displayed co-expression of podoplanin or VEGFR-3 on isolated CD34+ cells, suggesting possibility as a LSC marker. Among them, expression of podoplanin was continuously increased regardless of AML FAB subtypes. Therefore, we further isolated podoplanin+CD34+CD38- cells from MNCs by a FACS moflo and these cells were seeded on a matrigel dish to observe potential for colony forming unit (CFU). Within 2 weeks, we found that sorted podoplanin+CD34+CD38- cells have high potential for CFU compared to podoplanin-CD34+CD38- cell population, implying podoplanin may serve as a LSCs specific marker. Moreover, the sorted podoplanin+CD34+CD38- cells quickly produced leukemic mouse with stable engraftment of human blasts as well as severe phenomenon such as human myeloid blast infiltration, suppression of erythropoiesis and expanded size of spleen. Conclusion. We for the first time demonstrated that LEC markers, podoplanin, was highly expressed and enriched on CD34+CD38- cells in AML and also found that these cell populations can function as LSCs *in vivo* and in vitro. This data with novel marker could suggest some clues to develop therapeutic strategies as well as to investigate a relationship between lymphatic proteins and LSCs in AML.

Poster Board Number: F-2044

ESSENTIAL ROLE OF GPER-INDUCED PKA SIGNAL CASCADES IN THE SURVIVAL OF BREAST CANCER STEM CELL REVEALED BY PHOSPHOPROTEOMICS

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Human breast cancer stem cells (BCSC) identified recently have the ability to initiate neoplastic growth with differentiation and display higher resistance to commonly used treatment in breast cancer. Although some unique properties of BCSC have been deciphered, the molecular mechanisms are still poorly understood. Here, we applied the label-free technique to quantitatively compare the phosphoproteomic profiles between BCSC and non-cancer stem cells (nonCSC) derived from a xenograft of ER- (estrogen receptor) human breast cancer. We utilized a SEMI-strategy for multiplexed label-free quantitation combining pH/acid-controlled IMAC chromatography and LC-MS/MS for relative quantitation of site-specific phosphorylation. This strategy effectively increases the phosphopeptide quantitation coverage. Proteins with differential phosphorylation were mapped to multiple signaling pathways, including NOTCH, CDK/ERK and JAK-STAT pathways, which could potentially orchestrate the self-renewal and stemness of BCSC. We also found that BCSC displayed ~2-fold higher expression of GPER than non-CSC in 3 different xenografts of human breast cancer. GPER, a member of G-protein-coupled receptor (GPCR) superfamily has recently been shown to provide alternative signal transduction pathways induced by estrogens. We showed that stimulation of GPER by estrogens and anti-estrogen tamoxifen led to dose-dependent increases in the proliferation of breast cancer cells. This was accompanied by PKA-mediated Ser118 phosphorylation of BAD in ER negative breast cancer. GPER silencing via RNAi or the mutation of BAD pSer118 led to reduced survival and mammosphere forming capacity of BCSC, which implied the importance of GPER and its downstream PKA pathway to the maintenance of BCSC characteristics.

Poster Board Number: F-2045

DEREGULATION OF EARLY HEMATOPOIETIC GENES IN MOUSE CD41$^+$ LEUKEMIA CELLS EXHIBITING STEM CELL PROPERTY

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Mixed lineage leukemia (MLL) gene, which plays crucial role in embryogenesis and hematopoiesis, undergoes chromosomal translocation with over 60 different fusion partner genes in human leukemias. The resultant MLL-fusion oncoproteins are frequently implicated in infant leukemias with poor prognosis. It is believed that the existence of leukemia stem cells (LSCs) accounts for the high recurrence in leukemia patients. However, the identity of MLL-rearranged LSCs and the oncogenic transformation mechanisms remain unclear. It is thus crucial to identify the MLL-rearranged LSCs and develop targeted therapy for complete disease remission. Our study aims to investigate the development of LSCs mediated by epigenetic alterations, which was shown in MLL-rearranged leukemogenesis. We focus on MLL-EEN fusion protein, which causes an onset of acute myeloid leukemia. We observed an elevated expression of early hematopoietic markers Runx1 and CD41 in the murine bone marrow-derived Mll-Een expressing cells. The induction of Runx1 gene was further associated with an active histone modification, H4R3 asymmetric dimethylation, at the promoter region, presumably through the activity of protein arginine methyltransferase 1 (Prmt1). We isolated CD41+ cells and found that they have enhanced self-renewal ability in colony-forming cell assay and higher expression of genes (Bmi-1, Runx1, Tal-1 and Lmo2) that are associated with HSC activities, as compared with CD41low/- cells. This suggests that the CD41+ Mll-Een expressing cells acquire stem cell properties through activation of a stem-cell transcription circuit, leading to initiation of LSCs. On the other hand, knockdown of either Prmt1 or Runx1 gene by shRNAs in Mll-Een expressing cells led to significant decrease in cell number and colony number, indicating a reduction of *in vitro* self-renewal ability. Similarly, inhibition of Prmt1 by AMI-1 inhibitor treatment reduced both Runx1 and CD41 gene expression as well as the leukemogenic potential of Mll-Een expressing cells, suggesting a potential therapeutic approach to eradicate MLL-rearranged LSCs. To conclude, our study suggests a re-activation of stem cell program in CD41+ cells which might function as LSCs. It also provides novel insight into the therapeutic targeting of LSCs through potential epigenetic pathways in MLL-rearranged leukemia.

Poster Board Number: F-2046

IDENTIFICATION OF MICRORNAS INVOLVED IN LEUKEMIC STEM CELL PERSISTENCE IN ACUTE MYELOID LEUKEMIA; POTENTIAL LEUKEMIA THERAPY

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Only a minority of cells within acute myeloid leukemia (AML) is responsible for leukemia growth and maintenance. These leukemia-initiating cells share cell surface markers with somatic stem cells and have features of both self-renewal and differentiation which have given them the name "leukemic stem cells" (LSCs).

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Although 60-80% of AML patients achieve complete remission after chemotherapy, many patients experience a relapse which is thought to be caused by the survival of LSCs after chemotherapy. Eradication of LSCs is therefore necessary to cure AML patients. Both normal hematopoietic stem cells (HSCs) and LSCs co-exist in the bone marrow of AML patients and success of anti-LSC therapy relies on eradication of LSCs while sparing HSCs. For the development of these LSC-specific therapies the identification of molecules that are differentially expressed between normal and malignant stem cells is important. MiRNAs are small non-coding RNAs which regulate the expression of target mRNAs both at the transcriptional and translational level. These small RNAs are promising therapeutic targets since modulation of a single miRNA affects many pathways simultaneously. Our aim is the development of novel LSC specific microRNA-based therapeutic strategies by identification of microRNAs differentially expressed and functioning in LSCs and HSCs. We have identified several phenotypic differences between LSCs and HSCs that reside within the AML BM. These include aberrant expression of CLL-1, lineage markers, CD34 and CD45 expression, scatter properties, and recently the activity of aldehyde dehydrogenases. These newly identified LSC properties allowed the identification and purification of both LSCs and HSCs from AML patients and gave us the opportunity to identify miRNAs differentially expressed between LSCs and HSCs and between LSCs and leukemic progenitors all derived from the AML BM (n=6). MiRNAs with modulated expression in LSCs compared to HSCs were Mir-21, Mir-181a/b, Mir-551b, Mir-29b and Mir-196b. MiRNAs with modulated expression in LSCs as compared to leukemic progenitors were Mir-126, Mir-335, Mir-146a, Mir-1274a/b and Mir-1290. We have confirmed the expression patterns of several of the identified miRNAs by Q-RT-PCR in AML patients. In conclusion, we have identified several microRNAs that are differentially expressed between leukemic progenitors, LSCs and HSCs. These miRNAs might potentially maintain and characterize AML LSCs and may be valuable anti-leukemic microRNA-based therapeutics through their effects on proliferation/death in the absence or presence of chemotherapeutics.

Poster Board Number: F-2047

NOTCH1 SIGNALING DRIVES HUMAN T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA INITIATING CELL SURVIVAL AND SELF-RENEWAL

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Leukemia initiating cells (LIC) contribute to therapeutic resistance as a result of their capacity to accumulate mutations in pathways such as the NOTCH1 receptor signaling pathway that promote self-renewal and survival within specific niches. Activating mutations in NOTCH1 occur commonly in T cell acute lymphoblastic leukemia (T-ALL) and have been implicated in driving therapeutic resistance. However, the role of NOTCH1 activation in human LIC propagation and sensitivity to selective NOTCH1 receptor inhibition has not been established. Here we have established humanized T-ALL LIC mouse models transplanted with primary pediatric T-ALL samples. $CD34^+$ progenitors from $NOTCH1^{Mutated}$ T-ALL samples had higher leukemic engraftment and propagation capacity than $NOTCH1^{Wild\text{-}type}$ $CD34^+$ cells, suggesting that self-renewing LIC were enriched within the $CD34^+$ fraction of $NOTCH1^{Mutated}$ samples. Human NOTCH1 antibody (hN1 mAb) treatment reduced LIC survival and self-renewal in $NOTCH1^{Mutated}$ T-ALL LIC-engrafted mice, and also depleted a population of $CD34^+CD2^+CD7^+$ cells that harbored serial transplantation capacity. These results validate the utility of this humanized model for pre-clinical evaluation of potential treatments for T-ALL and underscore the therapeutic potential of selective NOTCH1 targeting in the eradication of T-ALL LIC. This treatment strategy could have broader application to other NOTCH1-driven malignancies including colorectal, ovarian, and breast cancers.

Poster Board Number: F-2048

NOVEL ISOFORM-SPECIFIC PROGRESSION SIGNATURE OF LEUKEMIC STEM CELLS IN CHRONIC MYELOGENOUS LEUKEMIA

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The purpose of the study is to generate a unique gene isoform expression signature derived from chronic myelogenous leukemia (CML) stem cell (CD34+CD38+Lin-) population that could be used clinically for prognostication and to monitor responses to therapy. CML represents an important paradigm for understanding the molecular evolution of cancer because it was the first cancer shown to be initiated at the hematopoietic stem cell level by BCR-ABL; the first cancer found to undergo blastic transformation following malignant reprogramming of committed progenitors; and the first target of molecular therapy (e.g. imatinib, dasatinib) based on targeting pathologically activated kinases. Previously described leukemia stem cells (LSC) signatures that predict disease status are based on gene expression and/or microarray analysis techniques. RNA-seq allows full transcriptome profiling of splice isoforms, which can provide critical information about the expression profiles of transcribed genes that cannot be detected by using microarray techniques (non-coding regions). Furthermore, some previous studies in CML use bulk tumor or CD34+ cell populations, whereas we examined the LSC population. No transcriptome-based signature has been described for disease progression in CML or other MPNs For this, total RNA derived from primary samples from blast crisis (BC) CML compared to chronic phase (CP) CML patients were sequenced using Illumina platform. The data analysis was performed using an algorithm developed in-house to identify isoform specific changes in where a unique LSC signature of progression was identified based on overall isoform expression of gene coding regions, CD molecule isoform expression, and ncRNA isoform expression. This analysis identified the top 20 splice isoforms, top 20 CD molecule isoforms, and top 16 ncRNA isoforms, that were differentially expressed and clustered together in BC CML versus CP CML Based on the unbiased clustering of isoform expression in BC versus CP, overall the splice isoforms and ncRNA isoforms showed the closest correlation between relative expression levels and disease status. Validations of these results by quantitative RT-PCR (qRT-PCR) in an expanded cohort of patients are currently on going by designing isoform-specific primers to confirm results of the RNA-seq analysis. These transcriptome based signatures allow the identification of LSC that correspond to disease progression from CP to BC in CML based on their unique expression profiles, which could be implemented in clinical trials and in patient diagnostics, prognostics, and evaluation of therapeutic response. This paves the way for patient-based personalized LSC medicine and could have broader application to other LSC-driven malignancies.

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STROMAL CELL DERIVED FACTOR 1 ALPHA AND MACROPHAGE MIGRATION INHIBITORY FACTOR INDUCED METASTATIC BEHAVIORS IN CXCR4 EXPRESSING COLON CANCER CELLS

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Metastasis of cancer cells is a major cause of death in cancer patients. The process of cancer metastasis includes the proliferation of the primary cancer cells, local invasion, intravasation and cancer cell survival in blood flow, extravasation and attachment to secondary organs, and metastatic growth in a new environment.In these mechanisms of cancer metastasis, CXC chemokine receptor 4 (CXCR4) and its ligand play an important role. Most of cancer stem cells express CXCR4 receptor and respond to a chemotactic gradient of its specific ligand SDF-1, suggesting that cancer stem cells probably represent a subpopulation capable of initiating metastasis. Stromal cell-derived factor-1α (SDF-1α, also known as CXCL12) is well- known as a ligand of CXCR4 and macrophage migration-inhibitory factor (MIF) is recently known. In many cancers including breast cancer, pancreatic cancer, and colorectal cancer, CXCR4/SDF-1α was studied in metastasis-related cancer behaviors, which include cell proliferation, adhesion, migration, and invasion. However, CXCR4/MIF has rarely been studied in the metastatic behaviors of colon cancer cells. In this report, the effect of SDF-1α or MIF was studied on cell cycle, cell proliferation, adhesion, and migration of CXCR4-expressing colon cancer cell lines. SDF-1α or MIF caused a decrease in the G0/G1 phase and an increase in the S and G2/M phases in SW480. In addition, SDF-1α or MIF caused an increase in cell proliferation, cell adhesion to fibronectin, and migration in HT-29 and SW480. AMD3100, a CXCR4 antagonist, attenuated these effects, which were increased cell proliferation, adhesion, and migration due to treatment of CXCR4-expressing colon cancer cells with SDF-1α or MIF. In conclusion, SDF-1α or MIF can affect the metastasis-related behaviors of CXCR4-expressing colon cancer cells.

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INTEGRATIVE OMICS STUDY ON EPIGENETIC MODULATORS IN COLORECTAL CANCER INITIATING CELLS DURING CANCER PROGRESSION

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Epigenetic alterations such as aberrant chromatin structure changes play important roles in the establishment of tumor suppressor gene silencing during tumorigenesis and metastasis. Heterogeneous epigenetic abnormalities at the levels of DNA methylation and core histone modifications are found to coexist with genetic alterations in most of the colorectal cancer specimens. The interactions between histone H3 lys9 methylation and the methylation of the nearby genomic DNA currently adapt a model whereby these two changes form a mutually reinforced silencing loop and contribute to the epigenetic inactivation of particular genes, such as tumor suppressor genes, cyclin-dependent kinase inhibitors, DNA repair genes, apoptosis mediators, nuclear receptors, transcription factors, and cell adhesion molecules, during the development of colorectal cancer cells. However, the function of the histone H3 lys9 methyltransferases in colorectal cancer initiating cells remains largely unclear. Euchromatin histone lysine N-methyltransferase 2 (EHMT2) is a major H3 lys9 methyltransferase which adds mono- and di-methyl groups to the lysine 9 of histone 3 in euchromatin regions. The result of EHMT2 activity ends in the transcriptional repression of its downstream targets, including epithelial cell adhesion molecule (EpCAM), an important molecule involved in preventing tumor invasion and metastasis. Here we not only use the existing colon cancer model, the ApcMin mouse, as a resource to provide materials for "omics" studies, we also use an Lgr5-EGFP-IRES-CreERT2 allele to manipulate epigenetic modulators, EHMT2, to provide colon stem cells and cancer initiating cells specificities to dissect their roles with a specific focus on the epigenetic regulatory networks in colorectal cancer progression *in vivo*.

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ISOLATION, CULTURE AND BIOLOGICAL CHARACTERISTICS OF TUMOR STEM CELLS IN HUMAN COLORECTAL CARCINOMA

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OBJECTIVE Colorectal carcinoma is a common malignant tumor with high occurrence and mortality rate. Here we report the isolation and characterization Colon Cancer Stem Cells from human colorectal carcinoma *in vitro*. METHODS Primary colorectal carcinoma cells were cultured in serum free conditions which give rise preferentially to self-renewal stem cell spheres. Cells capable of developing into tumor spheres were cloned by limiting dilution and clones from single-cell proliferation were isolated. The proliferation potential of cloned cells was tested using MTT colorimetric method. Cell surface marker including CD133, CD166, CD24, CD47, CD200, CD90, CD44 and EPCAM were analyzed by Flow Cytometry. The stem cell specific genes Oct4, Sox2, C-myc and Nanog were tested and quantified by real-time quantitative PCR. RESULTS Out of 4 primary colorectal carcinoma cell cultures, 3 grew into tumor spheres. From these primary cultures, 10 clones capable of unlimited self-renewal were isolated and expended by continuous subcultures. These clones are positive for surface marks CD166, CD47, CD44, and CD90, and negative for CD133, CD24, CD200 and EPCAM. Comparing with their original cultures, cell proliferation analysis showed the cloned cells have higher proliferation rate and express higher levels of stem cell-specific genes Oct4, Sox2 and C-myc. But Nanog was not finding in both cloned cells and their primary cultures. CONCLUSIONS We successfully get the Colon cancer stem cells from human colorectal carcinoma tissue, the cells can be self-renewing and indefinite proliferation, and express special cancer cell surface marker and gene. Correspondence: WANG Li-bin E-mail: wanglibin007@126.com (The research is supported by the National Natural Science Foundation of China, NO.81000958)

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THE HUMAN CANCER STEM CELL MARKER, CD166/ALCAM, CORRELATES WITH AGGRESSIVE AND RECURRENT COLORECTAL CANCER

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CD166, an adhesion molecule of the immunoglobulin-like family was identified as a colorectal cancer (CRC) cancer stem cell marker,

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capable of recapitulating the original human tumor in a xenograft model. However, despite its correlation with tumor-forming ability, its physiologic function in defining or driving cancer behavior is not known. To establish a role for CD166 expression in aggressive disease we undertook a systematic analysis of the CD166-expression level in all stages of CRC. CD166 expression was then correlated to stratified patient outcome data. Interestingly we determined that increased expression of CD166 correlated with node-positive disease and an overall decrease in survival in CRC patients. To further investigate a potential function for CD166 within the tumor microenvironment, we noted that CD166 expression is restricted to the lower portion of the normal intestinal stem cell niche, encompassing Paneth cells and Lgr5 progenitors, both Wnt responsive populations. To explore the possibility that CD166 may play a functional role in modulating the Wnt signaling pathway, we examined the intestinal stem cell niche of CD166 knockout mice. While these mice were viable and their intestines grossly normal, there was an overall decrease in villus height that accompanied a decrease in Lgr5 progenitors. Further, we found that $CD166^{-/-}$ cells within the intestinal crypt displayed reduced levels of Wnt signaling pathway and target genes. Together, these findings support an important role for CD166 in regulating communication between the stem cell and its microenvironment.

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HUMAN PROSTATE CANCER STEM CELLS HAVE A DISTINCT DNA METHYLATION PROFILE WHICH CHANGES UPON DIFFERENTIATION

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Prostate cancer is characterized by an accumulation of actively cycling and aberrantly differentiated luminal cells bearing markers of phenotypic, genetic and epigenetic heterogeneity. However, a small subpopulation of undifferentiated basal cells (<1%) persists within the cancer and is hypothesized to contain stem-like cells that give rise to luminal cancer cells. In order to dissect the epigenetic heterogeneity of prostate cancer, in the present study we analyzed DNA methylation patterns in pure subpopulations of primary cancer cells, on the basis of their differentiation status. GSTP1, an enzyme involved in intracellular detoxification, is highly expressed in prostate basal epithelial cells but is down-regulated in terminally differentiated luminal cells by a mechanism independent of DNA methylation. The GSTP1 promoter becomes frequently hypermethylated in prostate cancer, where the majority of cells have a luminal-like phenotype. By analyzing the DNA methylation patterns in basal and luminal like prostate cancer cells, it has been possible to unveil the origin of promoter hypermethylation in prostate cancer that ultimately leads to epigenetic dysregulation of the cancer cells. In established cell lines, GSTP1 was actively transcribed (measured by RT-PCR) and not hypermethylated (measured by pyrosequencing methylation assay) in basal-like cancer cells, while it was hypermethylated and down-regulated in luminal-like cancer cells. MACs selection of $Lin^-/CD31^-/CD24^+$ cells from prostate primary tissues highly enriched for cells with a luminal-like phenotype ($PanCytokeratin^+/GSTP1^-/Cytokeratin5^-/AR^+/Cytokeratin8^+$), while generation of prostate primary epithelial cultures gave rise to cells with basal phenotype ($PanCytokeratin^+/GSTP1^+/Cytokeratin5^+/AR^-/Cytokeratin8^-$). In luminal-like cells, GSTP1 was hypermethylated in cancer samples compared to benign controls. However, no significant hypermethylation of GSTP1 was found in the basal-like cells, where the gene was actively expressed in both benign and cancer samples. Lack of GSTP1 promoter methylation was also found in tumor xenografts, generated in $Rag2^{-/-}gammaC^{-/-}$ mice, from primary prostate cancer tissues. These xenografts do not undergo complete differentiation and show an intermediate phenotype expressing both basal and luminal markers, while retaining a stem-like basal population. Moreover, in BPH-1 cells, a fast cycling immortalized cell line, expression and promoter methylation of GSTP1 correlated with the differentiation status of the cells, being hypermethylated in more differentiated cells. Our results strongly indicate that within prostate cancer there is a subpopulation of undifferentiated stem-like cells that do not hypermethylate the GSTP1 promoter. We hypothesize that these cells can differentiate into luminal-like cancer cells, which down-regulate GSTP1 and hypermethylate its promoter as a consequence of aberrant proliferation. Future work will include determination of the mechanism of GSTP1 down-regulation in normal and malignant prostate epithelial differentiation, and whether this mechanism is shared with other genes frequently hypermethylated in prostate cancer.

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REGULATED PROTEOLYSIS OF TROP2 DRIVES MOUSE PROSTATE TUMORIGENESIS AND CELL SELF-RENEWAL VIA B-CATENIN SIGNALING

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Cancer cells share multiple characteristics with adult tissue stem cells such as self-renewal, proliferative capacity and differentiation. Several groups have identified Trop2, a type I trans-membrane glycoprotein, as a marker of stem/progenitor cells in adult tissues. In the human and mouse prostate, the Trop2 expressing subpopulation of basal cells (Trop2hi) has stem cell capacities such as self-renewal, tissue-regeneration and multi-lineage differentiation. Consistent with the relationship between stem cells and tumorigenesis, we have previously identified that Trop2hi cells are efficient targets for transformation in the prostate. Trop2 is highly expressed in various epithelial cancers and it is associated with unfavorable outcome. However, the functional role of Trop2 in stem cell self-renewal, its relation to transformation, and the molecular mechanisms by which Trop2 transmits signals to regulate these processes remain unclear. Gain and loss-of-function analyses in in vitro sphere and *in vivo* regeneration assays demonstrate that Trop2 is not only a marker of stem cells and advanced cancers but is also a regulator of self-renewal, tissue regeneration and tumorigenesis. We observe significantly elevated levels of Trop2 in human prostate cancer, lymph node metastases and in mouse models of prostate cancer. Over-expression of Trop2 induces mouse prostatic intraepithelial neoplasia (mPIN) *in vivo*. Trop2 is activated by regulated intra-membrane proteolysis (RIP), resulting in shedding of the extracellular domain (ECD) and release of the intracellular domain (ICD) to the nucleus. Trop2 controls stem/progenitor cell self-renewal, proliferation and transformation activity through two cleavage products (ICD and ECD) generated by RIP. RIP is required for Trop2 activity and cleavage mutants of Trop2 fail to enhance self-renewal in vitro. Secreted Trop2 ECD induces Trop2 cleavage and results in increased stem/progenitor cell proliferation. Trop2 ICD alone promotes self-renewal activity in an in vitro sphere assay and it initiates mPIN lesions in an *in vivo* regeneration assay. Trop2

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ICD is involved in a signaling cascade that is dependent upon β-catenin to promote self-renewal. The ICD, but not the ECD, interacts with β-catenin to induce downstream target gene expression, and genetic studies indicate that β-catenin is required for Trop2 self-renewal activity. While β-catenin loss does not affect baseline sphere-forming activity, we find that over-expression of Trop2 requires the presence of β-catenin to enhance progenitor activity in vitro. Our study provides evidence that Trop2 is a key regulator of self-renewal and transformation in the prostate and its function depends on its activation by RIP and downstream signaling through β-catenin . This suggests that heightened Trop2 promotes the stem-like properties of self-renewal and proliferative activity in prostate cancer. Delineating the molecular mechanism of Trop2 action allows development of therapeutic strategies applicable to not only prostate cancer, but other epithelial malignancies that exhibit increased Trop2 expression.

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DIRECT INTERACTION BETWEEN HUMAN PANCREATIC CANCER STEM CELLS AND STROMAL CELLS REMODELS EXTRACELLULAR MATRIX FORMATION FOR THEIR NICHE

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Human pancreatic ductal adenocarcinoma consists of highly heterogeneous tumor cells that form tumor microenvironment cooperatively with stromal cells. Pancreatic cancer stem cells have been reported to play critical roles in chemotherapy and radiotherapy resistance. However, little is known about the microenvironment of pancreatic cancer stem cells. To investigate formation of the pancreatic cancer stem cell niche, we isolated and characterized a novel subpopulation of pancreatic cancer stem cells, tentatively named KMC cells, from seven patients with disseminated pancreatic ductal adenocarcinoma. KMC cells required their direct interaction with stromal cells for their clonogenic growth. KMC cells had self-renewal capacity, differentiation ability, and in vivo tumorigenicity. Stromal cells formed a collagen microfibrillar network of extracellular matrix, which was transformed into a basal lamina-like structure by KMC cells. KMC cells induced mRNA expression of laminin and collagen type-IV, major components of basal lamina, but reduced that of collagen type-I and type-III and fibronectin-I, major components of extracellular matrix, in stromal cells. Moreover, laminin was enriched at the KMC cell-induced basal lamina-like structure. Our findings demonstrate that pancreatic cancer stem cells have the potential to remodel their microenvironment cooperatively with stromal cells, suggesting that targeting the KMC cell-induced *de novo* niche formation in cancer therapy may improve prognosis of patients with disseminated pancreatic ductal adenocarcinoma.

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ORIGIN OF CANCER STEM CELLS AND ITS SIGNIFICANCE IN SOLID OVARIAN TUMOUR PROGRESSION

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The present study was designed to critically analyze the cancer stem cell hypothesis and its relevance in metastasis. It has been widely reported that the surface epithelium cells of the ovary are the cells of origin of epithelial ovarian carcinoma as they undergo regular wear and tear during ovulation. To study the progression of tumour from the point of its development to the stage of metastasis and secondary tumour formation, an orthotopic rodent model was chosen. Spontaneously transformed surface epithelium cells of mouse ovary (ID8) were injected orthotopically in a C57/BL6J syngeneic host to mimic tumour development in patients. GFP was stably incorporated in the ID8 cells by lentiviral transduction to track them *in vivo*. It was found that typical hematopoietic stem cells (HSC) expressing the Lin-Sca1+c-Kit+ phenotype were recruited both to the primary tumour stroma and the ascitic fluid which is formed during the later stages of ovarian carcinoma. The percentage of these HSCs was comparable to the HSC population present in the bone marrow. It was observed that a small but significant fraction of tumour cells from the primary site and the secondary metastases gain the hematopoietic marker CD45 during tumour development. Within this population there also existed a sub population of cells expressing the hematopoietic stem cell markers CD34, c-Kit and Sca-1. Further investigation revealed that the expression of the hematopoietic phenotype in epithelial ovarian cancer cells was the result of cell-cell fusion which is probably occurring because of the close interaction between the hematopoietic system and the solid tumour. Apart from the hematopoietic phenotype these fused cells also over express CXCR4 which could render a superior invasive property to these cells when compared to the other cells. The existence of a stem cell compartment within this fused population could implicate fusion as one of the mechanisms responsible for the origin of cancer stem cells. We are trying to dissect this phenomenon and functionally characterize the cancer stem cell-like population to determine its significance metastasis.

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HARNESSING HUMAN ES BIOMARKERS FOR CANCER THERAPEUTICS

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Specific surface markers are valuable for monitoring the culture and behavior of stem cells and their status of differentiation. Many commonly used stem cell markers were directly adopted from phenotypic characteristics of other cell types with limited insight into the specific stem cells under study. Therefore, it is important to identify new and more markers of stem cells. Based on MALDI-MS and MS/MS analyses, we have found a close association of the expressions of glycosphingolipids (GSLs) on the surface of hESCs with differentiation. It is believed that the newly found glycans present in hESCs could be candidates for cancer detection and glycan-targeted therapy of human tumors. SSEA-3 is a pentasaccharide (2Galβ1′ 3GalNAcβ1′ 3Galα1′ 4Galβ1′ 4Glcβ1) and serves as the precursor of Globo H. SSEA-4 is the sialylated derivative of SSEA-3 (NeuNAcα2 -SSEA-3). On the other hand, Globo H, a known biomarker for cancers, was highly expressed uniquely in hESCs and its antibodies reacted with both Globo H and SSEA-3. Therefore, these markers for hESCs are perhaps the targets of therapy for cancers. We also showed that there was a striking switch in the core structures for globo- and lacto-series GSLs during differentiation of hESCs into embryoid body, neural progenitors, or endodermic cells based on MALDI-MS and MS/MS analyses, suggesting a close association of GSLs in hESCs with lineage-specific differentiation (PNAS 107 22564 2010; Stem Cells, 29: 1995 2011). More recently, we employed glycoproteomics and glycan analysis to analyze the glycoprotein/glycan expression patterns for hESCs. It was found that seven newly found surface glycoproteins were also highly expressed in breast cancer stem/initiating cells. For example, ESC02

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which is uniquely present in hESCs is highly expressed (at least 7 folds) in the paired cancer stem cell subpopulation of breast cancer from different patients versus non-CSC. When transduced with shESC02, the expression level of Sox2 in shESC02 cells was reduced to 40%, whereas no significant changes were seen in Oct4 and Nanog expression. In addition, gene knockdown of ESC02 expression leads to decrease in the self-renewal of both hESCs and mammary spheres of primary breast cancer from patients. Such ESC02 silencing in ESCs also results in developmental skewing toward endoderm/mesoderm differentiation in vitro and *in vivo*. Therefore, these results warrant the development of ESC02 as therapeutic agents for cancer, because knockdown of its expression fulfill the major requirements for new therapeutic agents of cancer: i.e. cell arrest and differentiation induction. Furthermore, shedding of ESC02 occurs to the medium after incubation with breast cancer cells. Thus, we had developed a platform to study the inter-relationship between stem cells and cancer and to develop new cancer detection method and glycan-targeted therapy.

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MESENCHYMAL STEM CELL INHIBITS LUNG CANCER CELL MIGRATION AND REDUCES XENOGRAFT TUMOR GROWTH WITH SUPPRESSING EPITHELIAL-MESENCHYMAL TRANSITION VIA A PARACRINE EFFECT.

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MSCs have been intensively associated with tumor progression due to the observation of MSCs being recruited and homing to tumor sites. They have been employed as novel cell-based delivery agents in cancer to deliver anti-cancer pro-drug to the sites of primary and metastatic tumors. The efficacy of such engineered MSCs in cancer therapy has recently been tested in preclinical trials. Apart from acting as cell-based drug deliverers, several reports have revealed direct involvements of MSCs in tumor progression and growth with controversy. For example, bone marrow-derived MSC (BM-MSC) has been reported to promote colon cancer growth by enhancing angiogenesis and inhibiting apoptosis of tumor cells. Direct culture of BM-MSC with breast cancer cells suggested that BM-MSC stimulate the epithelial to mesenchymal transdifferentiation (EMT) of breast cancer cells and enhance cancer metastasis. On the contrary, studies on liver cancer and lymphoma demonstrated that MSC inhibits tumorigenesis and metastasis. The controversy of these discoveries may suggest a cancer type-dependent manner of MSC-mediated pro- or anti-tumor effects. Li et al. recently reported the MSC-associated inhibition on lung tumor growth; however, how MSC mediates the regulation of lung cancer metastasis and recurrence is still unclear. In the present report, we analyzed the functional interactions between human BM-MSC and human lung cancer cells in an indirect Transwell co-culture system. We demonstrated that lung cancer cells were able to enhance MSCs mobility and recruit MSCs to the chamber containing lung cancer cells without direct cell-cell contact. On the other hand, the presence of MSCs effectively inhibited the migration, invasion, and wound healing capacity of several lung cancer cells, but not breast cancer cells. We also showed that the conditioned medium derived from MSC culture had the same effects on lung cancer cells in terms of migration, invasion and wound-healing mobility in a dose-dependent manner, meaning that some secreted molecules from MSCs in the conditioned medium modulates these effects. We further showed that the observed inhibition was not due to a reduced cell proliferation rate but associated with a repressed EMT pathway, as evidenced by the reduction of several EMT-related markers in lung cancer cells upon co-culturing with MSC or MSC-conditioned medium treatment. *In vivo* xenograft tumor formation experiments indicated that MSC had inhibitory effect on the subcutaneous tumor growth, as co-transplantation of BM-MSC with lung cancer cells reduced the tumor size in comparison to lung cancer cells-injected mice. Using cytokine array analysis, we further identified Oncostatin M as an important cytokine mediated the MSC-dependent regulation of lung cancer cell mobility. In conclusion, the present study demonstrated an inhibitory effect of MSC on lung cancer metastasis through repressing EMT pathway via Oncostatin M. The suppressive effect of Oncostatin M reported in this study may provide a direction of therapeutic method and drug development for cancer treatment in the future.

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CISPLATIN ENRICHES DRUG RESISTANT CD133 POSITIVE CELLS VIA ACTIVATION OF NOTCH SIGNALING IN NON-SMALL CELL LUNG CANCER

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Platinum-based combination chemotherapy is the first-line treatment for advanced non-small cell lung cancer, but tumor recurrence occurs in most of patients who have received this treatment. Recent evidences suggested existence of CD133-positve stem-like lung cancer cells is the cause of drug resistance and tumor recurrence. However, the correlation and between chemotherapy and regulation of CD133+ stem-like lung cancer cells have not been clearly determined. In the current study, we revealed that CD133-posive lung cancer cells identified by a human CD133 promoter-driven GFP reporter exhibited drug resistance and expressed stem-cell characteristics. Treatment of lung cancer cells with cisplatin was sufficient to result in enrichment of CD133+ cells, to induce DNA damage responses, to up-regulate levels of ABCG2 and ABCB1 which therefore increased resistance to doxorubicin and paclitaxel. We further found that cisplatin-induced enrichment of CD133+ cells was mediated by activation of notch signaling as judge by increased levels of NICD. Pre-treatment of cells with γ-secretase inhibitor, DAPT, remarkably reduced cisplatin-induced enrichment of CD133+ cells and increased the sensitivity to doxorubicin and paclitaxol. Ectopic expression of NICD reversed the function of DAPT on drug sensitivity. The similar effect was also observed in tumor-engraft experiments as cisplatin treatment significantly increased the number of CD133+ cells in tumor-engraft in nude mice. In contrast, intra-tumor injection of DAPT together cisplatin was able to significant decrease the number of CD133+ cells in the tumor-engraft of mice. Together, we demonstrated that cisplatin induces the enrichment of CD133+ cells leading to the increase of multi-drug resistance through the activation of Notch signaling, suggesting that combination of cisplatin with other molecules that can inhibit Notch signaling may reduce tumor recurrence in non-small cell lung cancer.

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THE ROLE OF ENDOSIALIN IN HUMAN MESENCHYMAL STEM CELLS IN LUNG CANCER

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Endosialin was emerged as a tumor marker in the past three decades. Studies indicated that only restricted cell types expressed endosialin, including cancer associated fibroblasts (CAF), pericytes and mesenchymal stem cells (MSC). We aimed to explore the function of endosialin in mesenchymal stem cells during cancer progression. We have generated endosialin knockout (KO) mice which containede a *LacZ* reporter gene. Endosialin expression patterns were analyzed by β-gal staining and immunohistochemistry (IHC) under physical and pathological conditions. Our data indicated endosialin expressed on mesenchymal cell during mouse embryonic development and decreased dramatically in adult mouse. In order to dissect the function of endosialin in MSCs under pathological condition, we transplanted lung cancer cells into endosialin KO mice. Based on IHC staining, our preliminary data indicated that endosialin expression in tumor mesenchymal cells was induced in mice bearing lung cancer cells. Our work suggests that endosialin-expressed MSCs play important role during cancer progression and we will further characterize the interactions between endosialin-expressed MSCs and cancer cells.

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ABERRANT E-CADHERIN EXPRESSION IN HUMAN/MOUSE EMBRYONIC STEM CELLS AND TUMORIGENESIS

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The loss of E-cadherin is a common hallmark of cancer that is associated with more aggressive tumour phenotypes. The E-cadherin to N-cadherin switch is well documented as part of the epithelial mesenchymal transition (EMT), which is linked to acquisition of a cancer stem cell (CSC) phenotype and metastasis. The dysregulation of E-cadherin in neoplasia and tumorigenesis (DENT) hypothesis suggests aberrant expression of E-cadherin contributes to abnormal proliferation of epithelial cells leading to neoplasm formation prior to EMT. Abrogation of E-cadherin in a weakly metastatic breast cancer cell line (MCF-7) induced increased proliferation and survival of cells which may contribute to neoplasm formation *in vivo*. Furthermore, the increased proliferation and survival observed in cancer cells translates to findings in mouse and human embryonic stem cells. We, therefore, suggest a role for E-cadherin in establishment of tumour cell mass and potential use of embryonic stem cells as a model system to study dysregulation of E-cadherin in cancer.

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ROLE OF MICRORNA-363 IN THE DEVELOPMENT AND PROGRESSION OF GASTRIC CARCER

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Gastric carcinoma is one of the most common malignancies and the second most lethal cancer worldwide. Although the diagnosis and treatment are improved and the incidence is decreased in gastric cancer. However, the prognosis of gastric cancer is poor. The underlying molecular mechanisms responsible for aggressiveness of gastric cancer have not yet been clearly elucidated. MicroRNAs (miRNAs) are involved in many biological functions such as proliferation, differentiation, apoptosis, and stress resistance. Recent studies showed that miRNAs could play oncogenic and tumor suppressive roles in tumorigenesis and could have diagnostic, prognostic, and therapeutic potential. In addition, miRNAs also play critical roles in the maintenance of stemness of cancer stem cells, or tumor-initiating cells. It was demonstrated that MBP-1 suppresses growth and metastasis of gastric cancer cells. MBP-1 is produced by alternative translation initiation from α-enolase gene. To identify miRNAs that have the potential to regulate gastric carcinogenesis through targeting MBP-1, we employed algorithms to search for the putative binding sites of miRNAs in the 3′ -untranslated region (3′ -UTR) of human MBP-1/α-enolase mRNA. Our *in silico* analyses revealed that there is a putative miR-363-binding site in MBP-1/α-enolase 3′ -UTR. After overexpression of miR-363 by adenoviral system, levels of MBP-1 and α-enolase were decreased in SC-M1 gastric cancer cells. Luciferase reporter gene activity was attenuated by miR-363 when the reporter plasmid containing MBP-1/α-enolase 3′ -UTR was transfected into SC-M1 cells. After deletion or mutation of the miR-363-binding site, miR-363 did not exert its suppressive effect on activity of reporter gene containing MBP-1/α-enolase 3′ -UTR. Therefore, miR-363 targets the 3′ -UTR of MBP-1/α-enolase mRNA. Results of trypan blue exclusion method and MTT assay showed that the growth and viability of SC-M1 cells were enhanced by miR-363 depending on down-regulation of MBP-1. Abilities of colony formation, migration, and invasion were also elevated by miR-363 in SC-M1 cells. However, the augmented effect of miR-363 on ability of gastric cancer progression was relieved by MBP-1 or α-enolase. SC-M1 cells dispersedly grew and had a spindle- and fibroblast-like morphology after overexpression of miR-363. Consistently, the levels of epithelial markers were decreased by ectopic miR-363 along with the enhancing expression of mesenchymal markers in SC-M1 cells. After transfection with antagomir-363, knockdown of endogenous miR-363 suppressed growth, viability, and tumor progression of SC-M1 cells through MBP-1. The xenografted tumor sizes of SC-M1 cells were enlarged by miR-363 in nude mice. However, the miR-363-enhanced tumor sizes were attenuated by MBP-1 overexpression. Metastatic nodules in lungs were also increased by miR-363 after intravenous injection with SC-M1 cells into NOD-SCID mice. The miR-363-promoted ability to form metastatic nodules in lungs was attenuated by MBP-1 overexpression. Besides SC-M1 cells, miR-363 also induced tumor progression of the other gastric cancer cells such as KATO III cells. Therefore, miR-363 induces growth and progression of gastric cancer cells through down-regulation of MBP-1.

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TELOMESTATIN INHIBITS GLIOMA STEM CELL SURVIVAL VIA THE DISRUPTION OF TELOMERIC G-QUADRUPLEX AND SUPPRESSION OF THE PROTO-ONCOGENE, C-MYB.

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Background: The development of effective therapies for glioblastoma multiforme (GBM) is a challenging endeavor due to the aggressive proliferation and the high migratory potential of this form of cancer. Recent studies have suggested the existence of a hierarchical organization of multiple heterogeneous cell populations in GBM having distinct tumor driving capacities. Now glioma stem cells (GSCs) are a critical therapeutic target of GBM. Methods: The effects of a G-quadruplex ligand, telomestatin (TMS), were evaluated using patient-derived GSCs, non-stem tumor cells (non-GSCs), and normal fetal neural precursors in vitro and in vivo. The molecular targets of TMS were determined by immunofluorescence *in situ* hybridization (iFISH) and cDNA microarray. The data was then validated by in vitro and in vivo functional assays, as well as by immunohistochemistry against 90 clinical samples. Results: TMS impaired the maintenance of GSC stem cell-state by inducing apoptosis in vitro and in vivo. The migration potential of GSCs was also impaired by TMS treatment. In contrast, both normal neural precursors and non-GSCs were relatively resistant to TMS. Treatment of GSC-derived mouse intracranial tumors reduced tumor sizes in vivo without a noticeable cell death in normal brains. iFISH revealed both telomeric and non-telomeric DNA damage by TMS in GSCs but not in non-GSCs. cDNA microarray identified a proto-oncogene, c-Myb, as a novel molecular target of TMS in GSCs and pharmacodynamic analysis in TMS-treated tumor-bearing mouse brains demonstrated a reduction of c-Myb in tumors in vivo. Knockdown of c-Myb phenocopied TMS-treated GSCs both in vitro and in vivo, and restoring c-Myb by overexpression partially rescued the phenotype. Lastly, c-Myb expression was markedly elevated in surgical specimens of GBM compared to normal tissues. Conclusions: These data indicate that TMS potently eradicates GSCs through telomere disruption and c-Myb inhibition, and this study suggests a novel GSC-directed therapeutic strategy for GBM.

Poster Board Number: F-2064

CELL-OF-ORIGIN DRIVES CANCER STEM CELL PHENOTYPE AND HETEROGENEITY IN A SPONTANEOUS MOUSE MODEL OF MEDULLOBLASTOMA

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Cancer stem cells (CSCs) are a subset of cancer cells with stem cell-like properties and the unique ability to initiate a tumor when transplanted. Emerging studies show that these cells are inherently more resistant to current therapies than bulk tumor cells and may be the underlying cause of tumor recurrence and treatment failure. Hence, it is hypothesized that novel therapies that target these cells will significantly improve cancer treatment. However, a major confounding factor that impedes efficient development such therapies is the apparent heterogeneity of CSCs even within the same clinical tumor type. While both stem and progenitor cells are thought to give rise to CSCs in the brain, it is unknown whether both cell types can be transformed by the same initiating event *in vivo*, how different cell of origin affects the tumor phenotype, and whether the CSCs derived from stem vs, progenitor cells have distinguishing characteristics. Here we show that a major contributing factor to CSC heterogeneity is the cell of origin and that CSCs from different origins have distinct molecular and cellular phenotypes. We show that transformation of neural stem or progenitor cells in Ptch+/- mice give rise to histologically similar but molecularly distinct tumors comprising three distinct subtypes (NG, GFD, GFI). The three CSC subtypes have distinct cellular phenotypes that are cell-intrinsic and stable over multiple passages *in vivo*. By activating the SHH pathway in a cell type specific manner, we demonstrate that transformation of neural stem cells give rise to the GFD subtype while transformation of EGL progenitors give rise to the NG subtype. We provide evidence that both NG and GFD subtypes can progress to the GFI subtype and that this progression is marked by a recurring chromosomal aberration, trisomy 6. Importantly, while the bulk tumor cells in all three subtypes express high levels of the SHH pathway genes, GFD CSCs are completely resistant to SHH pathway inhibition. In addition, the frequency and culture behaviors of the three CSC subtypes correlate with the aggressiveness of tumor *in vivo*. Importantly, we identified a gene signature that distinguishes the three subtypes at the bulk tumor level, independently demonstrating that the Ptch tumors are not homogeneous. Analyses of human medulloblastoma molecular subtypes with these signature genes show an unexpected correlation between mouse and human medulloblastoma subtypes and identify a patient group with poor prognosis. Together this study demonstrates that the cell of origin has a major role in determining the CSC phenotype and that critical pathways that sustain CSC proliferation and survival may be different from those that drives bulk tumor cell proliferation, a novel concept that has significant implications for developing targeted therapies in the future.

Poster Board Number: F-2065

CHARACTERIZATION OF BONE MARROW DERIVED-MACROPHAGES STIMULATED BY C6 GLIOMA STEM CELLS

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Tumor stem cells (TSCs) play important roles in tumor initiation and relapse, and are therefore considered as a bona fide target for tumor eradication. TSCs undergo self-renewal or differentiation in response to the specialized microenvironment called niche. Various types of cells, including vascular endothelial cells and stromal fibroblasts, have been reported to function as TSC niche. Tumor-associated macrophages (TAMs) are also a major component of tumor mass, and large numbers of TAMs are observed in many solid tumors, including glioma. However, it remains unclear how the interaction between TSCs and TAMs could contribute to tumor development. cDNA microarray analysis of highly tumorigenic C6 glioma side population (SP) and non-tumorigenic main population (MP) cells indicated that SP cells show significant upregulation of

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some cytokine genes related to monocyte recruitment and differentiation, suggesting that SP cells may have essential roles in TAM behaviors. To examine the effect of cytokines secreted from SP cells on differentiation of monocytes into TAMs, murine bone marrow-derived monocytes were incubated for 4 days with the supernatant of cultured SP cells. Freshly prepared monocytes showed an amoeboid morphology and a negligible number of round cells. C6 cell culture supernatant induced macrophage-like cell morphology in this culture. FACS analysis and immunocytochemical staining showed that these cells highly express macrophage markers CD11b (Mac1), F4/80, and CD68 when cultured with SP cell supernatant, suggesting that some secreted factors from SP cells can promote the differentiation of monocytes into macrophages. However, co-culture experiments *in vitro* showed that the SP supernatant-educated macrophages did not possess significant effects on the maintenance of SP cell numbers. In general, TAMs are known to exhibit several M2-associated tumor-promoting ability, including induction of angiogenesis and immune suppression. To investigate whether the SP supernatant-educated macrophages have M2-like functions, these cells were intracranially co-transplanted with SP cells into NOD/SCID mice. Contrary to our expectation, SP supernatant-educated macrophages did not promote, but rather suppressed the tumor growth initiated by SP cells, suggesting that functional properties of TAMs could not be fully induced by secreted factors alone from SP cells, and therefore the direct interaction between SP cells and macrophages might be essential for M2 polarization. Establishment of *in vitro* model systems of TAMs and understanding of the regulatory mechanisms underlying their behaviors could provide a clue to develop therapeutic strategies against tumor stem cells.

Poster Board Number: F-2066

DISHEVELLED 3, A COMPONENT OF CANONICAL WNT SIGNALLING INDUCES ONCOGENIC CONVERSION OF NEURAL STEM CELLS TO GLIOMA STEM CELLS.

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Malignant gliomas are one of the most aggressive and invasive tumors of the Central Nervous system (CNS) displaying frequent refractivity to treatment and commonly exhibiting poor prognosis. We have found that Dishevelled-3, a key mediator of Wnt signalling pathway is involved in conversion of non-tumorigenic neural stem cells to glioma stem cells. While glioma stem cells showed high expression of Dvl-3, its expression was marginally detected in lower grade glioma tumors and completely lacking in non-tumorigenic neural stem cells. Interestingly, we found that Knock-down of Dvl-3, inhibited Wnt signalling, which was evident from the down-regulation of β-catenin target genes and decrease in β-catenin mediated transcriptional activity. The Dvl-3 knocked-down glioma stem cells showed inhibition of cell proliferation as well as impairment in tumor forming potential. We also report high expression of Wnt ligands - Wnt1 and Wnt3a in higher grade gliomas. The Wnt1 and Wnt-3a knock-down cells showed lowered tumorigenic and invasive potential. Altogether, our study identifies Wnt3a as a potent glioma oncogene and thereby designates aberrant activation of Wnt signalling pathway as an important contributor in malignant behaviour of glioma stem cells. Further, we provide compelling evidence about role of Wnt signalling in promoting glioma cell proliferation, tumor development, progression and invasion. Our results indicate that identification and development of novel targets inhibiting Wnt activation may become one of the viable therapeutic options in management of therapeutically resistant gliomas.

Poster Board Number: F-2067

CD44 AND SSEA4 POSITIVE CELLS IN ORAL CANCER CELLS HAVE CHARACTERISTICS OF CANCER STEM CELLS<!--ENDFRAGMENT-->

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Background: Cancer is derived from a cancer stem cell (CSC), a tumor-initiating cell that has properties similar to those of stem cells. The identification and isolation of CSCs needs to be further improved; a variety of different cell markers have been reported in different kinds of tumors. Methods: CSC markers in human oral cancer cell lines were examined using flow cytometry. The stem cell properties of subpopulations expressing different markers were further assessed by in vitro sphere formation assay, stemness gene expression by qRT-PCR, drug resistance assay and its ability to form tumors in nude mice. Results: The HSC-4 human oral cancer cell line was composed of 25.3% of CD44 and SSEA4 double positive (CD44+SSEA4+) cells, and the subpopulation exhibited preferential expression of some stemness genes. CD44+SSEA4+ cells formed spheres more easily than CD44+SSEA4– and CD44–SSEA4– cells. *In vivo* xenograft experiments indicated that CD44+SSEA4+ cells showed the highest tumorigenic capacity as compared to other subpopulations or parental cells. These cells were more resistant to the anticancer drug cisplatin than compared to other cell populations. Conclusions: It is suggested that CD44+SSEA4+ cells are representative of CSCs in oral cancer.

Poster Board Number: F-2068

MOLECULAR PATHWAYS REGULATING GLIOMA STEM CELLS

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Defining the unique properties of glioma stem cells (GSCs) is a high priority for research aimed at elucidating the molecular mechanisms driving tumor initiation, and for developing therapeutic strategies that specifically target GSC populations. It is also possible that mutations in other signaling pathways promote progenitor self-renewal. Here, we investigate EGF-EGFR/Akt, p38MAPK pathways and hypoxia on GSCs. The survival of GSCs depends on the enhanced downstream signaling cascade (PI3K/Akt and Erk1/2 pathways) triggered by EGF ligand-binding and blockade of EGFR signaling eradicates the self-renewal capacity of GSCs, and the proliferation and differentiation of CD133 positive cells. Furthermore, p38 MAPK inhibition led to increase in EGFR expression by delaying internalization of the ligand bound receptor and reduced proliferation, enhanced CD133 expression, maintenance of the undifferentiated state, and decrease in cell death. In contrast, activation of the p38 MAPK pathway promoted reduced expression of EGFR and poor self-renewal. On the other hands, hypoxia is an inevitable outcome of the rapidly growing tumor outstripping its vascular supply, however it confers certain advantages on the tumor cells including therapeutic resistance. The hypoxia promotes the self-renewal of GSCs via hypoxia inducible factor-1α (HIF-1α) expressions, and restrained differentiation. Better understanding the molecular basis of the unregulated self-renewal of GSCs will allow us the design of more effective therapies, such as a combination of anti-angiogenic

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agent with drug targeting hypoxia, blocking EGF-EGFR axis and activation of p38MAPK.

Poster Board Number: F-2069

TRICHOSTATIN A-INDUCED CHROMATIN RELAXATION INCREASES ALTERNATIVE LENGTHENING OF TELOMERE PHENOTYPES IN TELOMERASE NEGATIVE IMMORTAL CELLS

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Telomere length is maintained by telomerase and alternative lengthening of telomere (ALT) pathway which bases on homologous telomeric recombination. Recent studies indicate that chromatin structure implicates in telomere length regulation. However, the relationship between ALT mechanism and chromatin state has not been elucidated. In this study, we monitored ALT phenotypes; ALT-associated PML bodies (APBs) and telomere sister chromatid exchanges (T-SCEs), after treatment of ALT cells with trichostatin A (TSA), an inhibitor of histone deacetylases which leads to chromatin decondensation. The frequencies of APB and T-SCE markedly increased in TSA-treated cells ($p < 0.005$) in which telomere length was unchanged and hTERT, a catalytic component of telomerase, mRNA remained undetectable. Introduction of telomerase into ALT cells suppressed TSA-mediated ALT phenotype induction. Our findings may suggest that TSA-induced chromatin relaxation facilitates telomeric recombination in ALT cells and that ALT mechanism competes with telomerase for the telomeres with a less compact structure.

Poster Board Number: F-2070

CELLULAR HETEROGENEITY CONTRIBUTES TO TUMOR STEM CELL MAINTENANCE IN C6 GLIOMA

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Tumors are composed of functionally distinct types of cells, which is called tumor heterogeneity. A hierarchy model of tumor stem cells (TSCs) has explained how this tumor heterogeneity arises; only a small subset of TSCs can initiate tumor growth through self-renewal and differentiation into non-tumorigenic progenies. Therefore, TSCs are considered as a promising target for tumor eradication. However, it remains unknown how TSC progenies contribute to tumor expansion; are they simply bystanders? In this study, we found non-tumorigenic TSC progenies could function as a niche to maintain TSC. Co-culture experiments of highly tumorigenic C6 glioma side population (SP) cells with non-tumorigenic main population (MP) cells revealed that SP frequency was retained in a MP-dependent manner, which required the close contact between SP and MP cells. Gene ontology analysis of cDNA microarray data comparing transcripts between SP and MP cells indicated that MP cells show significant upregulation of some essential basement membrane genes, including collagen IV and laminin. When SP cells were inoculated on culture dishes coated with ECL (entactin-collagen IV-laminin) matrices, SP frequency was highly retained at least for 6 days. These results suggested the potential plasticity of TSCs to differentiate into cells that compose their own niche in vitro. To investigate the significance of self-producing niches by TSCs *in vivo*, immunohistochemical analysis was performed using mice brain intracranially transplanted with GFP-labeled SP cells. Collagen type IV protein is specifically expressed around CD31(+) tumor vessels, and FACS analysis using tumor cell suspensions confirmed the approximately equal numbers of GFP(+) SP-derived and GFP(-) host-derived CD31(+) cells, suggesting that SP cells differentiate into vascular endothelial (VE) cells to compose the vascular niche containing ECM proteins. Although host-derived VE cells have SP phenotype, SP-derived VE mostly have MP phenotype with more accumulative and resistant properties for anti-cancer drugs, suggesting that SP-derived VE may confer some survival advantages as a drug barrier for tumor cells. In conclusion, our study provides important insights into the fundamental aspect of tumor development from TSCs and the functional multiformity of tumor vessels, which are yielded by heterogeneity of tumor cells.

Poster Board Number: F-2071

WHOLE-GENOME TRANSCRIPTOME ANALYSIS REVEALS MIR-29A TARGETS INVOLVED IN SELF-RENEWAL, APOPTOSIS AND EPIGENETIC REGULATION, INCLUDING CENTRAL COMPONENTS OF ACTIVE DEMETHYLATION AND MAINTAINANCE OF DNA METHYLATION STATUS

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MicroRNAs (miRs) play important roles in the control of self-renewal and differentiation, by controlling, both, the translation or degradation of its target transcripts. Recent studies revealed miR-29a as an important player in the regulation of hematopoietic (HSC) and leukemic stem cells (LSC). A preliminary database (microRNA.org) and literature search revealed that, in normal cells, the highest levels of miR-29a are found in T-cells, followed by B-cells and then (at comparable levels) by granulocytes, monocytes and CD34+ HSC; while in leukemic cells, highest levels are found in indolent Chronic Lymphocytic Leukemia (CLL), followed by aggressive CLL (at levels comparable to B-cells), by Mantle Zone Lymphoma (MZL), and then (at levels comparable to HSC), by B-cell and T-cell Acute Lymphoblastic leukemias (ALL) and Acute Myeloid Leukemia (AML). Overall, these studies associate to lower miR-29a levels to a more aggressive disease and a worst prognosis (for CLL, MZL and AML). In line, restoration of miR-29 in primary AML blasts or cell lines induces apoptosis and dramatically decreased tumor growth in xenograft models. Intriguingly, overexpression of miR-29 in mouse B-cells or HSC results, respectively, in the development of leukemia with B-CLL characteristics or, AML, by converting myeloid progenitors into self-renewing LSC. To shed further light on the role of miR-29a in self-renewal and leukemogenesis, we carried systematic identification of transcript targets. For this, synthetic pre-miR-29a, inhibitory anti-miR-29a and respective control molecules (Ambion) were independently electroporated into Jurkat cell (at 100 nM), using the Neon transfection System (Invitrogen). 48h post-transfection gene expression profile was obtained using Agilent Whole-Genome microarrays. Transcripts simultaneously down-regulated by the pre-miR and up-regulated by the corresponding anti-miR, were compared to the set of predicted targets showing evolutionary conserved miR binding sites (microrna.org) to identify confident targets. To identify pathways and biological processes modulated by the miR-29a, we used a Functional Annotation Tool (DAVID) and

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selected targets were validated using qRT-PCR. Among pathways with a statistically significant enriched number of miR-29a target transcripts, we identified: Apoptosis (FAS, BIRC2), WNT (WNT8B/16, FZD4/10, LRP6, TCF7L1), TGF-beta (TGFB3, ACVR2A/B, BMP8A, SMAD2, BMPR1A/B, LEFTY2,), Jak/Stat (LIF, LIFR, SPRY1) and cancer (MDM2, APC, NRAS, PTEN, PTENP1, RARB, FOS). Further inspection revealed central components of active demethylation (including TET1/2/3 and TDG) and maintenance of DNA methylation following cell division (DMNT3b). qRT-PCR confirmed the significant down-regulation of TET1/2/3 and DMNT3b after pre-miR transfection (pValue<= 0.05, n=3). Both, oncogene hypomethylation or tumor suppressor hypermethylation can lead to oncogenic transformation. For instance, mutations in the DNMT3A (leading to hypomethylation) or the TET family (leading to hypermethylation) are found in a significant fraction of myeloid disorders. Interestingly, Tet2 haploinsufficiency in the hematopoietic compartment leads to increased stem cell self-renewal and myeloproliferation, paralleling the effects of miR-29a overexpression. Our results indicate that miR-29a may have opposing effect depending on the context, and sheds light in its roles in normal and leukemic stem cells. Support: FAPESP, CNPq.

Poster Board Number: F-2072

STEM CELLS - ISSUES OF PLASTICITY, DIVERSITY AND CARCINOGENESIS.

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The phenomenon referred to as "stem cell plasticity" brings forth a number of issues and problems related to many biological and medical disciplines. Our presentation reviews some important questions and opinions concerning both stem cell plasticity and processes associated with stem cells with special emphasis on "tumor stem cells" and their potential role in carcinogenesis. A considerable component of our discussion is devoted to the phenomena associated with stem cell diversity; namely, to the relationship between phenotypic diversity and plasticity. Here we attempt to formulate general definitions of plasticity and diversity and show how these properties are interrelated. To illustrate the problem, we chose a theoretical model relevant to tissue regeneration. The results suggest some theoretical possibilities of tumor development and the reprogramming of tumor cells. It is our opinion that cell plasticity decreases with their stage of differentiation in time. However, plasticity will never be zero, since even highly differentiated cells retain a certain degree of plasticity. Generally speaking, as the number of stem cells decreases, the plasticity of the population decreases - the population has "low plasticity". If we assume that plasticity is a phenomenon applicable to all cells, including tumor cells, the development of a tumor stem cell may well result in a profound increase in plasticity. This highly plastic population may participate in processes of tissue regeneration. As we presume existence of a complementary relationship between the plasticity and diversity of stem cells, this highly plastic population may evoke a certain degree of diversity in all cells including tumor cells, and a population of low diversity cells may develop. This condition renders a new degree of diversity in all cells, and a new population of high diversity cells develops. On the grounds of this hypothesis, we predict that under certain circumstances, tumor stem cells may participate in tissue regeneration without initiating tumor development (low diversity cells), or with the imitation of tumor development (high diversity cells). Therefore, it may be presumed that in a certain transition period, tumor stem and progenitor cells may reprogram from the state of diversity (for example, from low to high to low) and stimulate the development of normal tissue. Here the interrelation between the plasticity and diversity of stem cells plays an important role in determining cell fate. Since the two phenomena cannot be temporally separated from each other but are indeed mutually exclusive. We view this situation in a manner similar to the principle of indefiniteness; cells must choose either plasticity or diversity, as both are not possible at the same time.

Poster Board Number: F-2073

A SUSTAINABLE BIO-BANKING OF HUMAN STEM-LIKE TUMOR-INITIATING CELLS DERIVED FROM CLINICAL PATIENT SPECIMENS

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Summary Banking of live tumor tissues is one functional alternative to maintaining the commercially available cancer cell lines on plastics. As most human tumors are composed of dynamically changing heterogeneous tissues, and only a rare pool of cancer stem cells in the total bulk tumor drive tumor formation, banking of purified stem-like tumor-initiating cells from patient specimens forms the important first step of any venture towards cancer therapies. Next, the discovery of novel bio-markers as well as their characterization and validation in cancer stem cells will be of great value for developing precise diagnostics and targeted therapeutics for human cancers. In concert with, the national bio-banking efforts through Office of Biorepositories and Biospecimen Research, we outline various strategies for utilizing the clinically annotated and functionally validated stem-like tumor-initiating cells. Benefits of progress in this direction will be evaluated in terms of laboratory analysis efficiencies, data modeling accuracy, improvements in patient diagnosis, quality of health care, and finally their Bio-bankonomics. Thanks are due to Drs. Lorenz Studer, Chris Park, Jackie Bromberg, and Gary Schwartz.

Poster Board Number: F-2074

NANOG/ZFP57 PATHWAY PROMOTES ANCHORAGE INDEPENDENT GROWTH OF HT1080 CELLS BY INDUCING IMPRINTED GENES EXPRESSION

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Since cancer cells and embryonic stem (ES) cells share several biological properties, it is possible that some genes expressed in undifferentiated ES cells might play important roles in growth of cancer cells. To explore this possibility, we first searched for such genes by examining the effect of ectopic expression of several genes involved in ES cell self-renewal on anchorage-independent growth of a human fibrosarcoma cell line, HT1080. As a result, we found that Nanog, a self-renewal promoting factor of ES cells, promotes anchorage-independent growth of HT1080 cells. This finding was confirmed by establishing HT1080/TRE-Nanog, where Nanog expression is induced by removal of doxycycline. On the other hand, Nanog failed to promote growth of HT1080/TRE-Nanog cells in adhesion culture. Using HT1080/Nanog shRNA cells, where Nanog shRNA expression is induced by addition of IPTG, we found that Nanog knockdown suppresses growth of HT1080 in soft agar, but shows no effect on growth in adhesion culture. These results suggest that Nanog is involved in anchorage-independent growth of HT1080. We next searched for Nanog target genes using HT1080/Nanog shRNA cells and identified another ES-specific

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transcription factor, Zfp57 as a downstream molecule of Nanog. In addition, we revealed that forced expression of Zfp57 restores suppression of growth in soft agar by Nanog shRNA. These results suggest that Nanog regulates anchorage-independent growth of HT1080 cells through Zfp57. Since Nanog transforms mouse fibroblast NIH3T3 cells (Piestun et al., 2006), we examined if Zfp57 has the same potential ability and found that overexpression of Zfp57 allows NIH3T3 cells to grow even in soft agar, suggesting that Zfp57 also has the oncogenic ability to transform NIH3T3 cells. Lastly, based on the previous reports that Zfp57 regulates expression of imprinted genes, including Dlk1 and IGF2, in embryo (Li et al., 2008) and ES cells (Quenneville et al., 2011), we examined if the same mechanism works in cancer cells. When we examined the relationship between the Nanog/Zfp57 pathway and imprinted genes, we found that both Dlk1 and IGF2 are downstream genes of Zfp57 and Nanog. Taken together, these results suggest that stem cell factors including Nanog and Zfp57 may induce imprinted gene expression to promote anchorage-independent growth of cancer cells.

Germline Cells

Poster Board Number: F-2075

DIRECT CONVERSION OF HUMAN SOMATIC CELLS TO MEIOTIC GERM CELLS BY GENETIC MANIPULATION

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Among the different diseases that can lead to infertility, poor gamete quality usually plays an important role and this is the reason because there is an emerging demand on donated sperm and eggs in fertility clinics. In the other hand, the study of human germ line development could lead to a better understanding of the diseases related to poor gamete quality and allow scientists to design treatments to improve it. However, the creation of in vitro models to study the insights of human germ line development is mandatory due to technical and ethical issues that make difficult to do it *in vivo*. Here, we report the direct conversion of two different human male somatic cell cultures to meiotic germ cells by the in vitro genetic overexpression of a combination of twelve selected key germ cell developmental regulators: PRDM1, PRDM14, LIN28, NANOS3, NANOG, DAZ2, DAZL, VASA, BOULE, STRA8, SCP3 and DMC1. The in vitro induced Germ Cells (iGCs) that we obtained resulted positive for alkaline phosphatase staining and expressed several male and female post-meiotic germ cell markers such as TNP2, PRM1, GDF9, ZP3 and ACROSIN, among others, in time-course experiments. Furthermore, we detected meiotic progression of our iGCs as shown by the observation of the correct assembly of the synaptonemal complex, and even formation of haploid cells in vitro, but in low frequency. Despite further research is needed in order to improve our results as shown by the high rate of chromosomal aberrations we found in our experiments, the in vitro generation of haploid iGCs from adult human cells by direct genetic reprogramming can be employed as a model to study the insights of meiosis, epigenetic reprogramming and other human germ cell development events in vitro. Moreover, these results represent a promise to be used in the treatment of infertile couples by replacing gamete donation, allowing them to be the biological parents of their child in the future.

Poster Board Number: F-2076

MAINTENANCE OF BIVALENT DOMAINS IN THE CHROMATIN OF EARLY DEVELOPMENTAL REGULATORY GENES IN MOUSE PRIMORDIAL GERM CELLS

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Pluripotency is dependent on maintenance of a proper epigenetic landscape. Bivalent domains, which are defined by the coexistence of a permissive mark (H3K4me3) and a repressive one (H3K27me3), are thought to play an important role in maintaining lineage plasticity by keeping developmental genes in a transcriptionally poised state for activation upon differentiation. While extensive studies have been performed in mouse embryonic stem cells (ESCs) demonstrating the important of bivalency in lineage commitment, only limited information is available regarding a role in the developing mouse embryo. Interestingly, primordial germ cells (PGCs) share a transcriptional profile similar to ESCs. However, unlike ESCs, PGCs are unipotent and unable to contribute to chimeras. We hypothesized that a divergent epigenetic signature would explain the disparate behavior of PGCs compared to ESCs. Due to the low number of PGCs present during development, nearly all evidence concerning the epigenetic status of PGCs is from immunofluorescent-based methodologies, which lack gene-level resolution. Using these approaches, it has been proposed that PGCs undergo a genome-wide reprogramming of histone marks, including H3K4me3 and H3K27me3, at E11.5. In order to properly investigate the effects of epigenetic regulation on gene expression we have developed a low cell number ChIP-qPCR protocol that allows for the analysis of histone marks using less than 10,000 cells per IP without the need for carrier chromatin. We used this protocol in conjunction with an Oct4::GFP reporter mouse line and FACS to isolate Oct4-positive PGCs from E11.5 embryos for epigenetic analysis. Surprisingly, we found that nearly all early developmental gene investigated (15 of 16), including several Hox genes, showed a strong enrichment for both H3K4me3 and H3K27me3 at their promoters in E11.5 PGCs. The bivalency that we detect at developmental regulators in E11.5 PGCs is remarkably similar to what we find in ESCs and correlates very well with gene repression. In contrast, important pluripotency regulators that are expressed in ESCs but silenced in PGCs, such as Klf4 and Upp1, are K4me3-only in ESCs but bivalent in PGCs. These findings have two potentially interesting implications: a) bivalency at early developmental regulators is not exclusively present in early embryos and ESCs, which activate these genes soon after differentiation, but is also found in E11.5 PGCs which will not activate them during gamete differentiation. These results raise the intriguing possibility that the germline may continuously mark developmental genes in a poised state for activation only post-fertilization, during development of the next generation. In agreement with this notion, bivalent domains have been found in the residual histones present at developmental regulators in mouse sperm. b) the genome-wide chromatin reprogramming reported to occur at E11.5 in PGCs does not appear to affect H3K4me3/H3K27me3 levels at developmental regulators. We hypothesize that H3K27me3 may represent an alternative epigenetic silencing mechanism to compensate for the loss of the DNA methylation and other repressive histone marks that occurs during this same time frame. We are taking advantage of our low cell number ChIP-qPCR protocol and emerging deep sequencing library amplification technologies to investigate the abundance of these and other histone marks at

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different stages of mouse germline development. Our most recent data will be discussed.

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EFFICIENT DERIVATION OF PLURIPOTENT STEM CELLS FROM NEONATAL MOUSE TESTIS USING SMALL MOLECULES

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The two first authors contributed equally in this work. Introduction: Spermatogonial stem cells reprogramme to a pluripotent state, embryonic stem (ES)-like cells *in vitro*, spontaneously. Less ethical concerns in addition to lack of immunogenicity, and their production without the need for genetic manipulation which currently used in iPS cell generation, had made these ES-like cells a novel opportunity in regenerative medicine. Given how low efficient and lengthy the process of ES-like cell production can be, we reasoned that the usage of small molecules as a tool for manipulating signaling pathways provide an opportunity to generate these cells in a more efficient and faster manner. Materials and Methods: To obtain a better robust derivation protocol, we have attempted to identify small molecules (SMs) that induce reprogramming of testicular cells in culture. Neonatal testicular cells were isolated and cultured with the presence of different SMs. The emerged ES-like colonies were further analyzed by immunofluorescent staining, Real time RT-PCR and differentiation ability. Results: We could identify a SM that could induce production of pluripotent cell line from two different mouse genetic backgrounds. Confirmation of pluripotency of the generated cell lines has been proved by *in vitro* spontaneous and direct differentiation toward cardiac and neural lineages. Also increased expression of pluripotency genes such as POU5F1and NANOG was seen along with reduction of germline specific markers. Furthermore, we have shown that these cells have the ability to expand and maintain under feeder and serum free defined medium and produce chimera after transplantation into host blastocysts. Discussion: Here we report that testicular cells of neonatal mouse can be reprogrammed into pluripotent stem cells through manipulating signaling pathways under chemically modified culture condition, regardless of animal genetic background. Future studies will determine whether generation of ES-like cells by usage of SMs could be applied to other mammals, especially human.

Poster Board Number: F-2078

IN VITRO TRANSFORMATION OF MOUSE SPERMATOGONIAL STEM CELLS

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Germ cell tumors (GCTs) are unique in that they exhibit diverse biological characteristics and pathological features. Although several *in vivo* GCT models are available, studies on GCTs are hampered because *in vivo* development of GCTs is time consuming and prevents a detailed molecular analysis of the transformation process. Here we developed a novel strategy targeting spermatogonial stem cells (SSCs) to transform germ cells in vitro. Lentivirus-mediated transfection of dominant negative Trp53, Myc, and activated Hras1 into an enriched SSC population caused tumorigenic conversion in vitro. Although these cells resembled embryonic stem (ES) cells, they were aneuploid and lacked Nanog expression, which is involved in the maintenance of the undifferentiated state in ES cells. Euploid ES-like cells were produced by transfecting the Yamanaka factors (Pou5f1, Myc, Klf4, and Sox2) into SSCs. Although these cells expressed Nanog, they were distinct from ES cells in that they expressed CD44, a cancer stem cell antigen. Both treatments induced similar changes in the DNA methylation patterns in differentially methylated regions of imprinted genes. Moreover, despite the differences in their phenotype and karyotype, both cell types similarly produced mixed GCTs upon transplantation, which were composed of teratomas, seminomas, and embryonal carcinomas. Thus, in vitro SSC transformation facilitates an analysis of the GCT formation process, and our results also suggest the close similarity between GCT formation and reprogramming.

Poster Board Number: F-2079

CHARACTERIZATION OF MOUSE MALE GEMLINE STEM CELL NICHE BY GENE EXPRESSION PROFILING USING LASER CAPTURE MICRODISSECTION

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Stem cells are tightly linked to their niche or microenvironment, which regulates their behaviors. The germline stem cell (GSC) niche in *Drosophila* and Caenorhabditis elegans containing easily identifiable GSCs has been extensively characterized on the molecular and genetic levels, revealing the importance of multiple signaling pathways and cellular processes. However, the mammalian GSC niche is less defined because unequivocal identification of GSCs has also not been achieved. In the mouse testis, a subset of primitive spermatogonia termed "undifferentiated spermatogonia" or "Aundiff" includes stem cells that constitute an as-yet-unidentified subpopulation. The Aundiff populations are located on the basal membrane within the seminiferous tubules, and are biased toward vascular network in the interstitial tissue. Differentiating spermatogonia left these regions and dispersed throughout the basal compartment of the seminiferous tubules. According to these findings the mammalian GSC niche is likely to be located around the vascular-associated regions. However, no specialized niche substructure and their cellular components within the seminiferous tubules have not been identified. To explore the detailed substructute and cellular components of the GSC niche, we initiated a comprehensive identification of the genes expressed around the vascular-associated regions within the seminiferous tubules. First, we isolated distinct cell-type populations between vasculature-associated regions and the other tubule bounding regions by Laser Capture Microdissection (LCM). Using these samples, we analyzed their gene expression differences by microarray. Until now, we verified 20 genes preferentially expressed around the vascular-associated regions by in situ hybridization. Within this subset, 3 and 17 genes were expressed in germ-line and somatic cells, respectively. Using their markers, we try to examine the spatial relationship with the Aundiff populations by immunostaining of whole mount testis. We anticipate that characterization of spatio-temporal expression patterns corresponding to putative stem cells will lead to greater understanding of stem cell-niche interactions.

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Poster Board Number: F-2080

EFFECTS OF CHROMATIN REMODELING FACTOR CHD1 ON *IN VITRO* DIFFERENTIATION OF MOUSE PREIMPLANTATION EMBRYOS

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Epigenetic regulation is considered to be one of the most important mechanisms by which changes in gene expression occur without changes in the underlying DNA sequence. Methylation of histones is a special process in epigenetic regulations that plays a dual role. Some activate gene expression and others inhibit it. Trimethylation of histone 3 lysine 4 (H3K4me3) has been shown to be a marker of the activation of gene expression. Chromatin remodeling factor Chd1 (Chromodomain Helicase DNA binding protein 1) is a protein belonging to the family of ATPase dependent chromatin remodeling factors. Chd1 recognizes H3K4me3, facilitates the competency of pre-mRNA maturation, and is required for the maintenance of mouse ES cell pluripotency. Chd1 is also necessary for the incorporation of the variant histone H3.3 in the absence of transcription into paternal pronuclear chromatin at fertilization in *Drosophila* embryos. However, the function of Chd1 in mammalian preimplantation embryos remains unknown. In the present study, the effects of Chd1 on in vitro differentiation of mouse preimplantation embryos were examined. To suppress the function of Chd1, siRNA targeting Chd1 was introduced to 1-cell embryos by microinjection. One-cell embryos were obtained by in vitro fertilization (IVF) and 3 hours after IVF 1-cell embryos were recovered and 5-10pl of 100μM siRNA was injected. Embryos were recovered for quantification of Chd1 and Oct4 mRNA 96 hours after microinjection. In embryos injected with siRNA, the amounts of Chd1 and Oct4 mRNA were confirmed by real-time RT-PCR (qRT-PCR). From the results, the amount of Chd1 mRNA was dramatically decreased from the 2-cell stage and the amount of Oct4 mRNA was decreased at the 4-cell and blastocyst stages. To analyze the phenotype of siRNA-injected embryos, we performed immunofluorescence staining of lineage-specific markers such as Cdx2 and Oct4 at the blastocyst stage. The siRNA-injected embryos have lost ICM and trophectoderm integrity and exhibited reduced expression of Oct4 and Cdx2. The findings implicated an important role of Chd1 after 4-cell stage of mammalian embryo development. It has been reported that Oct4 knockout mouse embryos are able to develop to the blastocyst stage but are not able to implantation. The expression patterns of Chd1 and Oct4 during preimplantation stage is experimentally confirmed by qRT-PCR. From the results, Chd1 mRNA levels dramatically increased during the 4- to 8-cell stage, peaked at the 8-cell stage, then gradually decreased after the 8-cell stage, and is scarcely detected in blastocyst stage, and Oct4 mRNA increased dramatically at the 4-cell stage and continues to the blastocyst stage. This suggests that initiation of Oct4 expression at the 4-cell stage depends on Chd1 and the inhibition of Chd1 at the 4- to 8-cell stage causes the decreased expression of Oct4 at the blastocyst stage. The findings implicated an important role of Chd1 after 4-cell stage of mammalian embryo development.

Poster Board Number: F-2081

AN ESSENTIAL ROLE OF A TUDOR DOMAIN-CONTAINING PROTEIN, KRIMPER, IN *DROSOPHILA* PIRNA BIOGENESIS

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A large fraction of the eukaryotic genome is occupied by transposable elements (TEs). TEs move around within the genome of their host cell, and therefore retain the potential to cause harmful mutations, chromosomal rearrangement, and other disorders. Hence, it is imperative for the cells to defend against the proliferation of TE in number of ways, as gene silencing. The silencing of TE in germline involves a subset of germline-specific small RNAs, PIWI-interacting RNAs (piRNAs), which themselves are mainly derived from TEs and antisense transcript of TEs. The biogenesis of piRNAs is not yet fully understood, although possible mechanisms have been proposed. Within one of the two pathways involved in piRNA biogenesis, the primary processing pathway, primary antisense transcripts transcribed from TEs and/or the piRNA clusters are processed to piRNAs, and are loaded onto Piwi protein, Aubergine (Aub) or Piwi. The complexes produced through this mechanism triggers another processing pathway, so called the amplification loop, wherein primary piRNAs recognize their complementary targets and amplify piRNA sequences. This amplification loop involves Piwi proteins, Aub and Argonaute 3 (Ago3), and also assisted by variety of Tudor domain-containing proteins. While Tudor domain-containing proteins are reported to play an essential role in piRNA biogenesis mainly through genetic studies, their precise molecular function has yet to be investigated. Here, we analyze the role of a Tudor domain-containing protein, Krimper (Krimp), in *Drosophila* ovaries. The expression level of piRNAs is significantly reduced in krimp mutant ovaries, suggesting its requirement in piRNA biogenesis. To understand the molecular function of krimp, we raised a monoclonal antibody against Krimp and performed biochemical analyses. We found that Krimp interacts with one of the Piwi proteins, Ago3, but not with the other Piwi proteins, Aub and Piwi. Moreover, Ago3 in krimp mutant ovaries appears to be free from symmetrical dimethyl arginines (sDMAs), which is known as an important post-translational modification to be associated with Tudor. Also, Ago3 that is co-immunoprecipitated with Krimp does not load piRNAs, and the amount of piRNAs loaded in Ago3 and Aub was reduced within krimp mutant ovaries. Together with observed disruption of Ago3 cellular localization among loss of Krimp function, our data indicate that Krimp regulates piRNA biogenesis through sDMA-independent direct association with Ago3, most likely in the piRNA amplification loop.

Poster Board Number: F-2082

A MITOCHONDRIAL PROTEIN DGASZ IS REQUIRED FOR PIRNA BIOGENESIS IN *DROSOPHILA* OVARIAN SOMATIC CELLS

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Drosophila piwi is essential for germline stem cell self-renewal. Previously, other and we have shown that endogenous Piwi associates with small RNAs of 25-29 nucleotides in length, which are now called as Piwi-interacting RNAs (piRNAs). piRNAs are mainly expressed from repetitive regions, such as retrotransposon and heterochromatic regions, in the *Drosophila* genome. Disruption of

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piRNA production or Piwi expression causes retrotransposon de-repression and loss of germline stem cells. These results together suggest that Piwi functions in retrotransposon silencing by associating with piRNAs originating from the repetitive genomic regions. However, little is known about the molecular mechanisms of piRNA biogenesis and Piwi-dependent retrotransposon silencing. Previous studies using an ovarian somatic cell (OSC) line indicated that piRNA biogenesis occurs in the cytoplasm, although the Piwi-piRNA complex functions in silencing retrotransposons exclusively in the nucleus. Some protein factors essential for piRNA biogenesis in OSC have been identified. RNAi screening revealed that the cytoplasmic Yb body components Armitage (Armi) and fs(1)Yb (Yb), both of which have an RNA helicase domain, are essential for piRNA biogenesis in OSC. Zucchini (Zuc), a mitochondrial phospholipase D (PLD) family member, was also identified as an essential piRNA biogenesis factor in OSC. To identify new piRNA factors, we continued our RNAi screen. To date, we screened approximately 100 genes. Of these, we demonstrate that knockdown of CG2183 resulted in a dramatic reduction in piRNA levels and led to derepression of retrotransposons. CG2183 is a *Drosophila* homolog of mouse germ cell protein with ankyrin repeats, sterile alpha motif, and leucine zipper (Gasz), which has been shown to be essential for piRNA biogenesis in mouse testes. However, the molecular functions of Gasz remain unclear. To elucidate the function of *Drosophila* Gasz (dGasz), we generated a specific monoclonal antibody against the protein. Biochemical analyses using anti-dGasz antibodies showed that dGasz mainly localizes at mitochondria, as does Zuc, and that dGasz interacts with Yb body components in OSC. These data suggest that mitochondrial functions are necessary for piRNA biogenesis and retrotransposon silencing in ovarian somas in *Drosophila*.

Poster Board Number: F-2083

ESTABLISHMENT OF *IN VITRO* CULTURED PIG SPERMATOGONIAL STEM LIKE CELLS USING ALTERNATIVE TEMPERATURE CONDITIONS

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Spermatogenesis initiated from a population of cells called spermatogonial stem cells (SSCs). Although several species, include mice and rat, SSC has been established in vivo, in vitro and *ex vivo* culture system, SSC from the domestic animal has not been cultured in vitro. In this study, pig SSC like cells were successfully isolated from neonatal pig, and cultured for long time using alternative culture medium and temperature. To isolate pig SSC like cells, several different media were used, and low and high temperatures were applied as the culture conditions. The cell clumps were appeared from neonatal pig testis cells under only GDNF, LIF, bFGF and EGF contained media conditions at 3 days after cells were seeded, and more number of clumps were identified in low temperature condition. Analyses of these cells with SSC markers identified in other species revealed that alkaline phosphatase staining was positive, and the strong expression of PGP9.5, Nanog, Gata4, DBA, Vasa were determined with reverse transcriptase polymerase chain reaction and western immuno blot analysis. In conclusion, this study showed a useful purification method of pig SSC like cells from neonatal pig testis without any physical damage or selection with antibody or gradient density of germ cells, and these cells were stably proliferated in GDNF, LIF, bFGF and EGF contained medium at low temperature culture conditions up to 100 days. Therefore, suggested method for establishment of pig SSC like cells may contribute to study of spermatogenesis occurred in vitro, and to study of domestic animal biotechnology.

Poster Board Number: F-2084

ELUCIDATION OF RNA SILENCING MECHANISM MEDIATED BY PIWI IN *DROSOPHILA*

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Members of the Argonaute family of protein play important roles in RNAi and RNAi-related RNA silencing pathways in various organisms. Argonaute proteins contain PAZ and PIWI domains, and according to the peptide sequences they fall into two classes, Argonaute (AGO)- and Piwi-subfamilies. In *Drosophila*, five Argonaute genes are encoded in the genome. Of those, AGO1 and AGO2 belong to the AGO-subfamily. Biochemical and genetic studies revealed that AGO1 and AGO2 function in miRNA- and siRNA-mediated RNA silencing, respectively. Argonautes cleave mRNAs complementary to small RNAs through their slicer activity of PIWI domain. Other three *Drosophila* Argonautes, Piwi, Aubergine, and AGO3, are categorized as the Piwi-subfamily members and mainly expressed in gonadal cells. *Drosophila* Piwi is an essential nuclear protein for germline stem cell self-renewal. It has been shown that Piwi is required for retrotransposon silencing in gonadal somatic and germline cells. Previously, others and we have shown that Piwi associate with piRNAs produced from retrotransposons and heterochromatic regions. Disruption of piRNA production or Piwi expression causes retrotransposon de-repression. These findings indicate that the Piwi-piRNA complex is involved in retrotransposon silencing by controlling their mobilization in the germline. In contrast with siRNA-mediated RNA silencing, Piwi possessing mutations at two aspartic residues (D614, D685) required for the slicer activity does not cause a loss of the retrotransposon silencing activity in a *Drosophila* ovarian somatic cell line (OSC). Therefore, the molecular mechanisms of Piwi-dependent retrotransposon silencing are largely elusive. To understand the molecular mechanisms of Piwi-dependent retrotransposon silencing, we performed RNAi screening using OSCs in which the piRNA biogenesis and piRNA-dependent retrotransposon silencing occur. This approach enabled us to identify several piRNA biogenesis factors such as Armitage, fs(1)Yb and Zucchini (Zuc). Importantly, we also found that three other genes are required for retrotransposon silencing but not for the piRNA accumulation, suggesting that these factors are involved in the silencing step in the piRNA pathway. To characterize their biochemical functions, we are engaging on producing specific antibodies against retrotransposon silencing factors to analyze their interactions. The outcomes of these experiments will be presented at the meeting.

Poster Board Number: F-2085

MOLECULAR SIGNATURES AND CULTURE OF TESTICULAR GERM CELLS IN COMMON MARMOSET CALLITHRIX JACCHUS

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Common marmoset (Callithrix jacchus) has been considered as an excellent model for human neurogenic disorder because of its great resemblances with human. Although genetic modified marmoset has been created by injecting lentiviral transgene into the embryo, the transgene is incorporated randomly. In order to create transgenetic animal with targeted gene modification, the germ cells has been considered as another option. This is because germ

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cell is responsible for spermatogenesis, which is a fundamental process to pass genetic information from one generation to next. Germline stem cell lines have been successfully established from spermatogonial stem cells in rodent, and most recently in rabbit. This has provided conventional means to investigate the germ cell properties. However, unlike mouse, the information in the molecular characteristics of the primate germ cell was poorly understood, which cause the challenge of developing primate germ cell culture. To understand the properties of the primate germ cell, we have examined gene expression of germ cell markers and DNA methylation status according to the previous studies on rodent or human. Bisulfite genomic sequencing revealed the marmoset-specific epigenome status in pluripotency factors distinct from that in mice; while evolutional similarity between marmoset and mice in other genomic loci analyzed was also found. RT-PCR and immunohistochemistry of germ cell marker demonstrated gene expression profile distinct from those in the mice, too. We then described a novel protocol to cultivate marmoset testicular germ cells in the form of cell aggregation called "testicular sphere". Investigation of alkaline phosphatase activities showed that marmoset testicular germ cells could be maintained more than 8 weeks by this method. RT-PCR also verified that our method enables us to cultivate marmoset germ cell up to 30 days while keeping the expression of various germ cell markers. Moreover, cryo-preserved testicular germ cells can also form sphere. To confirm the ability of genetic modification, we have transducted lentiviral transgene into our spheres. We assume that our method can provide easy manipulation and assessment of primate germ cell in culture. Therefore, this study holds the promise to for cultivating primate germ cell, which take us a step close to generate transgenetic marmoset with specific gene-targeting.

Poster Board Number: F-2086

EFFECTS OF INCUBATION TEMPERATURE ON PRE-PUBERTAL BOVINE SPERMATOGONIAL STEM CELL PURITY, PROLIFERATION, AND STATE OF DIFFERENTIATION

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Pluripotent stem cells have numerous potential applications in biomedicine; however, isolating and maintaining stem cells in culture has proven problematic for some sources. Our objective was to determine the effects of isolation method and temperature on purity, proliferation and differentiation of pre-pubertal bovine spermatogonial stem cells (SSC). Three experiments were conducted: experiment A (Exp. A), determined effects of standard SSC isolation method followed by incubation at 37°C or 41°C; Exp. B, determined effects of a modified SSC isolation method, only mechanical versus mechanical and enzymatic digestion, followed by incubation at 37°C or 41°C; and Exp. C, determined effects of incubation temperature (37°C or 41°C) with mouse embryonic fibroblasts (MEF) feeder cells on SSC after cryopreservation. In this study we demonstrate that simplifying isolation method and increasing incubation temperature yields a greater level of proliferation while maintaining the highest number of undifferentiated SSC. Furthermore, we show that cryopreservation has little effect on the growth and differentiation of SSC after thaw and culture.

Poster Board Number: F-2087

DIFFERENT SIGNALINGS FOR PLURIPOTENCY ACQUISITION IN EACH STAGE OF PRIMORDIAL GERM CELLS

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Germ cells are committed cells that have unipotent differentiation capacity, giving rise to only gametes. However it is known that primordial germ cells (PGCs) converted to pluripotent embryonic germ (EG) cells in appropriate culture conditions. This conversion could be recognized as the process of pluripotency acquisition from unipotent germ cell lineage. This will be a good model to analyze how to acquire pluripotency and how PGCs control the balance between pluripotency and unipotency. First, we tried to establish efficient culture condition that induces pluripotency from PGCs. Through the screening of chemical compounds including epigenetic modifiers such as TSA and 5AZA, we found highly efficient culture condition of PGCs to EG cells by adding 3 inhibitors. In this culture, EG cells were generated from PGCs at ~15% of efficiency. They are MEK inhibitor (PD325901), GSK-3β inhibitor (CHIR99021) and TGF-βR inhibitor (A83-01). Because the combination of MEK and GSK-3β inhibitors is well known as 2i for reprogramming, our 3 inhibitors combination is designated as 2i+A83. On the other hand, AKT involved in the signaling required for the acquisition of pluripotency. In the presence of 2i+A83, AKT signal showed the additive effect for obtaining pluripotency. It is indicated that the activation of AKT is independent from 2i+A83 signal for EG cell formation. In the absence of bFGF, whereas AKT induced EG cells, but 2i+A83 did not. Therefore, AKT is essential signal for conversion to EG cells, while the effect of 2i+A83 is supportive. Different from the data of E11.5 PGCs described above, EG cells was generated in the presence of LIF and 2i from E8.5 PGCs. In the development of PGCs, there are some genome wide changes during E8.5 to E11.5 such as transcriptional quiescence and epigenetic shift from H3K9 methylation to H3K27 methylation. We are now identifying the relation between these genome wide changes in PGCs and pluripotency acquisition at the molecular level.

Poster Board Number: F-2088

OCT4 SUMOYLATION NEGATIVELY REGULATED BY SENP1 MODULATES DRUG SUSCEPTIBILITY OF TESTICULAR GERM CELL TUMORS UNDER HYPOXIC CONDITIONS

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Testicular germ cell tumors (TGCTs) associated with poor prognoses are insensitive to chemotherapies and express low levels of OCT4. Hypoxia is known to induce drug resistance in TGCTs, however the mechanism underlying the hypoxic effect on OCT4 protein level and drug resistance in TGCTs remains to be determined. Here we

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demonstrate that hypoxia reduces the level of OCT4 expression and increases the resistance of embryonal carcinoma (EC) cells to cisplatin and bleomycin. Furthermore, we show that the loss of OCT4 expression under hypoxic conditions can be triggered by sumoylation. And this sumoylation was regulated by SUMO1 and SUMO1/sentrin specific peptidase 1 (SENP1), as overexpression of SUMO1gg (the active form of SUMO1) under hypoxia not only increased the level of sumoylated OCT4 (Su-OCT4), but also decreased the stability of OCT4 protein in EC cells. In addition, overexpression of SENP1 in EC cells reduces the level of Su-OCT4 induced by SUMO1gg overexpression, thereby maintaining OCT4 expression levels and enhancing the sensitivity of EC cells to cisplatin in vitro and *in vivo*. Site-specific mutagenesis experiments further revealed that the sumoylation of OCT4 occurred at the lysine 123 residue, as overexpression of HA-OCT4-K123R effectively reduced the level of Su-OCT4 under hypoxic conditions. In conclusion, we demonstrate that hypoxia reduces OCT4 expression levels in EC cells which increases drug resistance. The loss of OCT4 was resulted from hypoxia-induced sumoylation, and overexpression of SENP1 in EC cells can counteract the suppressive effect of hypoxia, restoring OCT4 stability and drug sensitivity. These findings suggest that SENP1 may be a potential therapeutic target for drug-resistant TGCTs.

Hematopoietic Cells

Poster Board Number: F-2089

THIRD TRIMESTER C-KIT(+) HUMAN AMNIOTIC FLUID STEM CELLS CAN MIGRATE TO THE HAEMATOPOIETIC SYSTEM OF THE FETAL MOUSE AFTER PRENATAL XENOTRANSPLANTATION

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Introduction: Human amniotic fluid stem (hAFS) cells are a unique subgroup of human AF-derived stem cells that are isolated by C-Kit immunoselection. The haematopoietic potential of AFS cells was demonstrated in adult immunodeficient mice, where mouse AFS cells engrafted in the bone marrow. In this study, we explored engraftment after *in utero* xenotransplantation (IUT) of human AFS cells in immune competent mice. Materials and methods: Ckit+ AFS cells were isolated from cultured AF or fresh AF collected in the 3rd trimester (34-35 weeks of gestation). Human AFS cells (10,000 to 60,000) were transplanted into the peritoneal cavity of every fetal mouse in pregnant CD1 mice (n=6, at E14). The peripheral blood of recipient mice was analysed 4 weeks after birth for engraftment by flow-cytometry using anti-human beta2-microglobin antibody. PCR and immuno-staining for anti-human mitochondrial antibody was performed on neonatal tissues collected 6 weeks after birth. The bone marrow was assayed for colony-forming cells (CFC). Results: The average live birth rate after IUT was 67%. Flow-cytometry analysis showed higher levels of human cells in the mice peripheral blood (5.1 vs 1.7%), liver (4.3 vs 3.4%), spleen (1.2 vs 0.5%) and bone marrow (2.3 vs 0.2%) after transplantation of fresh hAFS cells compared to cultured hAFS cells ($p<0.05$). CFC assays in mouse bone marrow demonstrated bone marrow cells of human origin, which was confirmed by PCR. Human cells could also be found in the liver of transplanted animals in the immunohistochemistry study. Discussion: We have demonstrated *in utero* xenotransplantation of hAFS cells into the bone marrow of adult immune competent mice; fresh hAFS cells appear to function better than cultured hAFS cells. Further work is needed to confirm the haematopoietic potential of these cells in secondary recipients.

Poster Board Number: F-2090

HUMAN AMNIOTIC FLUID STEM CELLS DERIVED FROM SECOND TRIMESTER HAVE HIGHER HEMATOPOIETIC POTENTIAL THAN FROM THIRD TRIMESTER

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Introduction: Human amniotic fluid stem cells (hAFS) can be isolated by Ckit selection and have been proved having mesenchymal and haematopoietic potential. The amniotic fluid can be collected easily from amniocentesis from 15 weeks to term. In this study, we investigated the different potential of hAFS derived from second and third trimester in all aspects. Material and methods: Ckit+ hAFS cells were isolated from second (amniocentesis at 15-22 weeks) or third trimester (Cesarean section at 35-39 weeks of gestation) pregnant women. Mesenchymal lineage cells were cultured in the adherent conditioning plates. Adipogenesis and osteogenesis differentiation from Ckit+ hAFS cells were performed in both groups. Haematopoietic linage cells were cultured in the suspension with essential cytokines then colony-forming assay was performed to see the different hematopoietic colonies. PCR and immunohistochemistry were also arranged for pluripotent markers. Results: The average percentages of Ckit+ hAFS cells were 1.7%, and 1.4% in second trimester and third trimester samples (n=10, each group). There was no significant difference in cell growth doubling time or in differentiation ability while culturing cells in the mesenchymal condition. In colony observational study, the number of the haematopoietic colonies was more in second trimester samples than third trimester ($p<0.05$). The hAFS cells from both groups were positive for Oct4, Klf-4, c-myc, NANOG; but negative for Sox-2. The immunostaining for the cells also showed higher percentage of positive staining cells in second trimester ($p<0.05$). Conclusion: Our study demonstrated the different potential of the hAFS cells derived from second trimester and third trimester. Both groups have similar mesenchymal stem cells characteristics, but second trimester amniotic fluid cells have higher haematopoietic stem cells potential than third trimester ones.

Poster Board Number: F-2091

OPTIMISATION OF IN-VITRO EXPANSION PROTOCOLS FOR HUMAN HEMATOPOIETIC STEM / PROGENITOR CELLS AND IN-VITRO ERYTHROPOIESIS

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Hematopoietic stem cells (HSC) are the paradigm for stem cell therapy. During the last decades, HSCs from cord blood have emerged as a true alternative to bone marrow-derived HSCs. Cord blood derived HSC have some advantages compared to bone marrow-derived HSC: i)fast procurement ii) less stringent HLA matching criteria iii) non-invasive collection with no pain for the donor. The major disadvantage is the low number of HSC in a single CB unit compared to bone marrow aspirates, which is a high obstacle for a widespread clinical use of CB HSCs. One strategy to overcome this limitation is the attempt to increase the number of

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HSC *in vitro*. Proof-of-principle experiments, demonstrating the successful expansion of HSC *in vitro*, have already been performed, and several different factors, which enable HSC expansion, have been described so far. We are performing a standardized comparison of different compounds with respect to their efficacy for in-vitro expansion of hematopoietic stem and progenitor cells. Different expansion conditions are analysed by multi-parameter immunophenotyping, by LTC-IC assays and by in-vitro differentiation assays. As one of our major research interests is in-vitro erythropoiesis, HSC expansion is the first step to generate massive amounts of erythroid progenitor cells. Thus, expanded HSCs are subjected to terminal erythroid differentiation in the presence of erythropoietin, and the effects of different cytokines added during this HSC expansion step on erythroid cell numbers, hemoglobin accumulation and production of enucleated erythrocytes are compared. The results give new insights into the regulation of erythroid proliferation and differentiation. This work was supported by the EC grant no. FP7-223011 HYPERLAB.

Poster Board Number: F-2092

CHARACTERISTICS OF HEMATOPOIETIC AND MESENCHYMAL STEM CELLS ISOLATED FROM CRYOPRESERVED HUMAN PLACENTAL TISSUE

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OBJECTIVES: Potential of banking placental tissue as a source of hematopoietic stem cells and mesenchymal stem cells is very important for regenerative medicine. MATERIALS: Human term placenta and cord blood were cryopreserved by conventional methods. Freshly enzymatically isolated cells and cultured stromal cell were analyzed by flow cytometry using CD34, CD45, CD31, CD90, CD105, CD73, CD14, CD133, HLA-ABC antibodies (all from BD, USA). RESULTS: We have show the possibility of cryopreservation of placental tissue for the reason to obtain viable hematopoietic progenitor cells (HPC) and mesenchymal stem cells (MSC). The FACS demonstrated the presents of population both CD34lowCD45low and CD34hiCD45low cells. The relative percentage of SSClow cells in population of CD34+CD45low isolated from cryopreserved placental tissue was 85,6% (range: 68,9-96,5) that significant higher than from native tissue 60,8% (range: 44,9-75,6). The percentage of population CD34+CD45lowSSClow cells (HPC) of all hematopoietic cells (CD45+ cells) from native and cryopreserved placental tissues weren't difference and were 0.66% (range: 0,36-1,05) and 1,11% (range: 0,18-2,82) respectively. Also we first show that HPCs from placental tissue were hirer level of expression of CD90 and CD31 compare to HPC form cord blood. Furthermore FACS of the stromal cultured cells from cryopreserved human placental tissue demonstrated that cells had a MSC phenotype, namely, CD90+CD105+CD73+HLA-ABClow CD45-CD34-CD133-CD14- and adipogenic and osteogenic potential *in vitro*. CONCLUSION: Developed method of cryopreservation human placental tissue allow save and obtain hematopoietic and mesenchymal stem cells.

Poster Board Number: F-2093

ABNORMALITIES IN NOONAN SYNDROME AND NOONAN/JMML HUMAN IPS-DERIVED HEMATOPOIETIC CELLS

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Noonan syndrome (NS) is a genetic developmental disorder caused by deregulation of the RAS/MAPK pathway. Germ-line mutations in *PTPN11*, which encodes SHP-2, a key component of the RAS/MAPK pathway, cause 50% of NS, while somatic mutations in this gene account for 35% of juvenile myelomonocytic leukemia (JMML). Children with NS and specific *PTPN11* mutations are at increased risk for developing JMML, inferring that certain SHP-2 mutants result in abnormal proliferation and cell maturation in hematopoietic lineages. The molecular mechanisms resulting from SHP-2 deregulation that lead to these abnormalities remain largely unexplored. Our aim was to elucidate signaling pathway alterations in myeloid progenitors in NS and NS/JMML using human induced pluripotent stem cells (hiPSC) derived from patients with those disorders. We established two hiPSC lines as controls and eight hiPSC lines with germ-line *PTPN11* mutations: Y63C and E76D in NS samples and D61H and G503C in NS/JMML samples. We differentiated these hiPSCs into hematopoietic lineages using specific cytokines. Hematopoietic populations (surface markers: CD33, CD14, CD11b, CD71, CD235a and CD41) in these samples were determined with flow cytometry. Proliferation and apoptosis were determined with Ki67 and annexin V staining, respectively. To check clonogenic capacity, cells were seeded on methylcellulose with specific cytokines to obtain CFU-GMs and CFU-Es. We assessed two clinical criteria used routinely for definitively diagnosing JMML: hypersensitivity to GM-CSF and absence of BCR-ABL fusion gene by FISH. Using RT-PCR and western blotting, we analyzed the levels of *STAT5* and a panel of specific miRNAs (miR181, miR128a, miR20a, miR17, miR106, miR223 and miR15a) in the mixed population and CD33+ myeloid progenitors. We observed an increase of myeloid progenitors (45%) and monocytes (18%) in NS/JMML compared to controls (15% and 8%, respectively). In addition, we observed an increase in the size and the total number of colonies in NS/JMML. The NS/JMML lines showed hypersensitivity to GM-CSF responding at 0.1 ng/ml, to which controls were not responsive. BCR-ABL fusion was absent. Apoptosis rates were similar in all samples, while the proliferation rate was increased approximately eight fold in CD33+ NS/JMML cells. These changes in proliferation in CD33+ NS/JMML cells correlated with increased expression of *STAT5* compared to control CD33+ myeloid cells. After screening of miRNAs associated with differentiation of hematopoietic cells using RT-PCR, we observed that the expression level of miR223, a specific regulator of granulocyte/monocyte precursors, and miR15a, related with STAT5 pathway, were increased 20 and 6 fold, respectively, in the myeloid population with mutations of *PTPN11,* while the expression levels of the other miRNAs assessed were comparable to controls. This study provides the first model of leukemia using hematopoietic cells differentiated from hiPSCs. Moreover, these studies provide new insights about *PTPN11*-driven JMML, revealing up-regulation of STAT5, miR223 and miR15a. These findings provide potential novel molecular targets for treating JMML, which remains a lethal disorder. Our future work will be directed at determining the

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upstream regulators and downstream effects of increased STAT5, miR223 and miR15a. We are also attempting to use the NS/JMML iPSC-derived hematopoietic progenitors to develop a transplant mouse model of JMML.

Poster Board Number: F-2094

HUMAN FETAL LIVER-DERIVED HAEMATOPOIETIC STEM CELLS FOR THE TREATMENT OF BLOOD DISORDERS

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Aim: Genetic diseases such as the thalassaemias, can lead to permanent tissue damage in early life. To address this issue, haematopoietic stem cells (HSC) have been proposed as cellular candidates for intra-uterine stem cell therapy (IUSCT) because of their ability to functionally engraft into host tissue and potentially effect rescue. Here, we study the use of fetal liver-derived haematopoietic stem cells (flHSC) for such applications. We hypothesised the fetal liver to be a rich source of HSC. Due to the primitive nature of these cells, we expect a high capacity for engraftment. In addition, we expect the cells to be amenable to gene augmentation, raising the possibility of autologous therapy or adjuvant gene therapy by serving as gene delivery vehicles. Methods: Mid-gestation fetal liver tissue (15 - 20 weeks) enzymatically dissociated and subject to flow cytometry analysis. Subsequently, CD34 positive cells were selected through magnetic activated cell sorting (MACS), and multi-lineage HSC differentiation capacity was confirmed on methycellulose cultures. flHSC were then compared against umbilical cord blood (UCB)-derived HSC for their capacity for gene augmentation at various multiplicities of infection (MOI). The capacity of lentivirally-engineered cells for multi-lineage repopulation was evaluated in sub-lethally irradiated NOD/SCID/Il2rg-/- mice. Finally, flHSC were injected pre-natally to evaluate potential application for IUSCT. Results and Discussion: HSC markers (CD34 and CD133) were found to be highly expressed in fetal livers, at a mean frequency of 9% to 0.5% ($p<0.0001$) of that in UCB. Following MACS, CD34+ cells demonstrated ability for multi-lineage differentiation in methylcellulose semi-solid cultures. Transduction with lentiviruses resulted in more than five-fold increased expression of transgene in flHSC over UCB-HSC (64% vs 12%, $p<0.01$) at MOI of 20, suggesting fl-HSC to be highly efficient gene delivery vehicles. Functional engraftment of transduced cells was demonstrated following injection, confirming ability of these cells to repopulate and reconstitute the haemopoietic system. Finally, IUSCT was performed on a total of 20 mice with evidence of human cell chimerism. Conclusion: Mid-gestation fetal liver tissue represents a rich source of HSC, which are capable of integration into host tissue. These HSC were found to be highly amenable to gene augmentation, indicating them to be a highly efficacious target cell type for gene delivery. Our *in vivo* results confirm the feasibility of this approach, and the experiments to study their application for the treatment of blood disorders in an IUSCT setting are ongoing.

Poster Board Number: F-2095

THE CRITICAL ROLE OF SOX17 IN THE DEVELOPMENT OF EARLY HEMATOPOIETIC PROGENITORS FROM HUMAN EMBRYONIC STEM CELLS

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Human embryonic stem cells (hESCs) have become valuable models for the study of early human development, *in vitro* analysis of lineage commitment and differentiation, and regenerative therapy. However, efficient development and isolation of useful cells from hESCs still remains a significant challenge. To find genes which can be manipulated in order to efficiently produce hematopoietic cells from hESCs, we screened known hematopoietic regulator genes by transducing them into hESC-derived CD34+CD43-CD45- endothelial cells enriched in hemogenic endothelium induced by an embryoid body (EB) method. Among the genes tested, only SRY (sex determining region Y)-box 17 (SOX17), a gene encoding a DNA binding transcription factor of the SOX (SRY-related HMG-box) family, promoted cell growth and expanded CD34+CD43+CD45low cells co-expressing endothelial makers such as VE-cadherin. SOX17 was specifically expressed in CD34+CD43-CD45- endothelial cells but not in CD34+CD43+CD45- pre-hematopoietic progenitor cells (HPCs) or CD34+CD43+CD45+ HPCs. SOX17-overexpressing cells formed sphere-like colonies on OP9 bone marrow stromal cells and generated few hematopoietic progeny. Upon inactivation of SOX17, however, they efficiently gave rise to hematopoietic cells, including erythroid and myeloid cells, suggesting hemogenic potential. Global gene expression analyses revealed that the CD34+CD43+CD45low cells expanded upon overexpression of SOX17 are hemogenic endothelium-like cells developmentally placed between CD34+CD43-CD45- endothelial cells and pre-HPCs. Of interest, overexpression of SOX17 also reprogrammed both pre-HPCs and HPCs into hemogenic endothelium-like cells. Genome-wide mapping of SOX17 by a ChIP-chip analysis revealed that SOX17 directly activates transcription of key regulator genes for vasculogenesis and hematopoiesis. Notably, depletion of SOX17 by short hairpin RNA in CD34+CD43-CD45- endothelial cells severely compromised their hemogenic activity. Recent studies of Sox17 conditional knockout mice have shown that Sox17 plays an important role in the fetal hematopoiesis in the yolk sac and fetal liver as well as in the maintenance of fetal and neonatal, but not adult, HSCs. Overexpression of Sox17 has also been demonstrated to confer fetal HSC characteristics to adult hematopoietic progenitors. Our findings demonstrate a critical role of SOX17 in priming hemogenic potential in hemogenic endothelial cells, and thus suggest a novel function of SOX17 in hematopoietic development. SOX17 could be a novel target to be manipulated to enhance the induction of hematopoietic cells from hESCs

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CLOSER SIMILARITY OF DNA METHYLATION PROFILES IN HUMAN BLOOD-DERIVED IPSCS TO ESCS THAN IPSCS DERIVED FROM HUMAN ADULT FIBROBLASTIC CELLS

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Studies on different models of mouse and human reprogramming support the notion that cell lineage-specific epigenetic modifications are not completely erased in iPSCs. Different levels of residual and tissue-specific DNA methylation persist in iPSCs derived from different somatic cell types. It is unclear, however, how these epigenetic marks influence the reprogramming and subsequent differentiation of iPSCs to various somatic cell types. Last year, we published a genome-wide DNA methylation analysis using Infinium HumanMethylation27 Beadchip that interrogated ~27,000 methylation sites (Chou et al., 2011). We observed that blood cells have a methylome closer than age-matched fibroblastic cells to ESCs, which plausibly explained the observation that human blood cells are easier to be reprogrammed than fibroblastic cells by either retroviral or episomal vectors. However, due to the small number of sites, the 27K chip has limitations to identify epigenetic memory marks (e.g. tissue-specific DNA methylation patterns that are shared by somatic cells and their derivative iPSCs but different from ESCs). We then utilized the HumanMethylation450 Beadchip that interrogates ~450,000 methylation sites, including 96% of CpG islands, island shores and the regions flanking them, for second round of methylation analysis. Moreover, we additionally included 4 non-integrating iPSCs (derived by episomal vectors) from blood (to B-iPSC) and mesenchymal stromal cells (MSCs) (to M-iPSC) of the same healthy individual. All the iPSCs are extensively characterized and 3 are fully sequenced. Consistent with previous studies, our new data demonstrated that DNA methylomes of B-iPSC and M-iPSC were globally similar to ESCs, and very different from that of blood cells or MSCs. At the global level, the methylome of B-iPSC group showed closer similarity to human ESCs than that of M-iPSC group to ESCs. Moreover, we also identified 449 differential methylated sites that were present uniquely in blood cells or MSCs and remained uniquely in B-iPSC or M-iPSC, but absent in ESCs. Currently, we are examining the effects of these epigenetic memory marks on the differentiation potential of B-iPSC and M-iPSC to either blood cells or MSCs. We also examined the methylation status of an incompletely silenced gene in fibroblast- or other adherent cell-derived iPSCs, C9orf64 (Ohi et al., 2011). Supporting the reported result, our data revealed that the C9orf64 promoter was hypermethylated in ESCs but not in either type of somatic cells. M-iPSC lines exhibited intermediate methylation in the C9orf64 promoter, representing incomplete epigenetic reprogramming. However, three blood-derived iPSC lines from the same individual (from whom the M-iPSC lines were derived) resembled that observed in ESCs and carried hypermethylated status of the C9orf64 promoter. Our findings suggested that while incomplete reprogramming of methylation states in somatic cells can exist, different cell type of origins can behave differently, even they are treated under the same reprogramming method and from the same individual. Our current study reveals a closer global methylome of blood cells to ESCs and less extensive epigenetic memory in blood-derived iPSCs when compared to fibroblast-derived iPSCs. Therefore, iPSCs from blood cells are not only easier to derive from a more accessible cell source with less mutations, but also likely in better quality, as they resemble more closely to human ESCs epigenetically.

Poster Board Number: F-2097

GPI-80 DISTINGUISHES TRANSPLANTABLE HUMAN FETAL HEMATOPOIETIC STEM CELLS FROM MULTIPOTENTIAL PROGENITORS

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Hematopoietic stem cells (HSCs) are responsible for the lifelong maintenance of all the cells of the blood system. Self-renewal and differentiation of HSCs is tightly regulated by the complex interplay of extrinsic and intrinsic factors that work together to establish and maintain the HSC pool. However, the regulatory mechanisms necessary to maintain the stemness of human HSCs are not well understood, in part due to lack of specific surface markers to purify HSCs for molecular studies. In order to facilitate a detailed study of the human long-term HSC, we performed genome-wide gene expression analysis comparing undifferentiated CD34+CD38-CD90+ human fetal hematopoietic stem and progenitor cells (HSPCs) and their progeny (CD34+CD38-CD90-) and attempted to identify novel markers that would further help to purify the long-term HSCs. Expression analysis identified 58 differentially expressed candidate HSC surface markers, including GPI-80 (Vnn2), a GPI-anchored surface protein recently described in neutrophil adherence and migration. Flow cytometry revealed that only a subpopulation of CD34+CD38-CD90+ HSPCs expressed GPI-80. Strikingly, GPI-80 expression segregated HSPCs into two functionally distinct populations; NSG mice (NOD/Shi-scid IL2rgamma chain null mice) transplanted with CD34+CD90+GPI-80+ cells (herein noted as GPI-80+HSPCs) displayed robust, multilineage human hematopoietic reconstitution, whereas CD34+CD90+GPI-80- cells (GPI-80-HSPCs) showed little to no hematopoietic engraftment. Furthermore, *in vitro* assays showed that while both populations were multipotent and generated myeloerythroid, B, and T lymphoid cells in culture, only the GPI-80+HSPCs demonstrated the ability to self-renew on mouse mesenchymal stem cell stroma, whereas GPI-80-HSPCs displayed loss of self-renewal and rapid differentiation. Strikingly, lenti-viral mediated shRNA knockdown of GPI-80 expression lead to loss of all undifferentiated HSPCs in culture, indicating that GPI-80 is also functionally required for self-renewal *in vitro*. To identify regulatory mechanisms that define the engraftable HSCs, we selected differentially expressed genes between GPI-80+ and GPI-80- HSPCs and assessed each set for enrichment of Gene Ontology (GO) categories associated with specific biological processes. There was no difference in genes regulating lineage differentiation, consistent with the finding that both populations are multipotent. However, GPI-80+HSPCs were enriched for genes related to cell migration, suggesting that these cells possess unique mechanisms required for HSC-niche interactions. These data identify GPI-80 as a novel marker for human fetal HSCs and a critical regulator for HSC engraftment and self-renewal.

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DIFFERENTIATION OF MONOCYTIC LINEAGE CELLS FROM HUMAN IPS CELLS BY USING A SERUM AND FEEDER FREE CULTURE METHOD

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It is important to establish a simple and solid method of hematopoietic differentiation from human induced pluripotent stem cell (iPSC) for researches using disease-specific iPSC and regenerative medicine. In this regards, we have already established a serum- and feeder-free solid culture method for stepwise hematopoietic differentiation from iPSC, especially into erythroid and myeloid lineages (PLoS One. 2011;6:e22261). Monocytic lineage cells, namely monocytes, macrophages and dendritic cells play key roles not only in immune response and defense against pathogens, but also in various pathological conditions. In this study, we established a serum- and feeder-free (matrigel-coated) culture method for differentiation of monocytic lineage cells from iPSC. CD34+CD43+ hematopoietic progenitor cells were induced by 3 cytokine-cocktail steps as follows: first step (4 days); BMP4 in mTeSR1 medium for mesoderm differentiation, second step (2 days); VEGF, SCF, FGF2 in StemPro-34 medium for hemangioblast differentiation, and third step (7 days); SCF, IL-3, Flt3L, TPO, M-CSF in StemPro-34 medium. Thereafter, CD14 positive monocytic cells induced by GM-CSF, M-CSF and Flt3L were sorted (Day15-22). These CD14-positive monocytic cells were differentiated into both macrophages by using M-CSF and dendritic cells by GM-CSF and IL-4 in StemPro-34 medium. When we compared some characteristics such as the morphology, surface maker and the results of functional assays including chemotaxis assay and cytokine assay, the iPSC-derived monocytic lineage cells shared the same characteristics with those *in vivo*. This method for monocytic lineage cell differentiation is useful both for regenerative medicine and for the analysis of the pathogenesis of immune disorders and the discovery of new drugs for these disorders, because this is the robust and simple method and mimics for monocytic lineage cell differentiation *in vivo*.

Poster Board Number: F-2099

ANALYSIS OF THE 1727 UMBILICAL CORD BLOOD IN LIAONING CORD BLOOD BANK CHINA AND QUALITY ASSESSEMENT OF 23 THAWED CORD BLOOD UNIT

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Objective:Cord blood(CB) are increasingly being used for hematopoietic transplantation, Alought for cord blood transplantation, 1 to 2 HLA mismatched cord blood was not the obstacle for clinical transplantation, Cord blood bank still need quite a few units for clinical searching. Nowadays the doctor looking for the suitable CB based on the total nuclear cells(TNC) and/no CD34+CD45dim+cell number and HLA typing, and now the CFU-C is becoming the important parameters which . Because the cord blood must cryopreserved, The limited TNC number and cryopreserved impair will delayed inplantation or caused graft failure. Analysising the TNC, CFU-C and CD34+CD45dim+ and assessment the viable cell and hemapoietic stem cell of the thawing sample can giving the reliable parameters to clinical . In cord blood bank, the quality sample are the segment tube attaching the bag and a E.P. tube. We also must know if those sample can reflect the cells in the bag. Method:We analysis the 1727 samples which cryopreserved over 1 year in Liaoning Cordblood bank, and analysis the affected facters on TNC, and the relation among TNC, CFU-C and CD34+CD45dim+. We also evaluated the vaibility and functional capacity from 23 cord blood sample which cryopreserved after 24 months . the thawed sample from segments, tubes and bags were assayed respectively. Result: Parameters of 1727 CB in Liaoning Cord Blood Bank Blood Volume:96.62± 20.41ml(48.00ml~208ml);TNC before centigue:12.51 ×108±4.40 ×108(8.00 ×108~26.5 ×108);TNC after centigue:9.88×108±2.54×108(7.80×108~26.1×108) Cryopreserved Volume46.41ml±3.84ml(37.00ml~61.00ml) Total CD 34+CD45+dim2.76×106±1.85×106(0.11×106~17.63×106):Tot al CFU-C:11.05×105±7.72×105(0.00×105~42.54×105)TNC recovery:80.53%±8.79%(5.95%~133.15%); Relation among TNC, CFU-C and CD34+CD45dim+ ,TNC after centigue got the positive relatation with Blood Volume(r=0.43), Total CD34+CD45+dim(r=0.49) and Total CFU-C (r=0.47), P<0.01; the results of assessement of 23 thawed cord blood unit ,the trypan blue in bag, in EP tube and in segment tube was 83.04%±6.21%,78.9%±6.56%,80.43%±6.01%,al l lower than before freezing(P<0.01), TNC(×108) in bag, in EP tube and in segment tube were 8.58±2.90,7.78±2.28,7.97±2.73 were lower than before freezing(p<0.01), Total CD34+(×106) in bag, in EP tube and in segment tube were 3.02±1.88,3.12±1.85,3.62±3.07; Total CFU-C(×105) in bag, in EP tube and in segment tube were 12.25±5.03,7.04±3.91,12.19±6.11 the CFU-C in the EP tube wes lower than in the bag before freezing (P<0.01) and in segment tube(p<0.05).Conclusion:1727 CB samples can used in 6, 5,4 matched HLA patients who weight lower than 50kg, 41.7kg, 35.7kg. TNC is positive relation with total CFU-Cand total CD34+CD45dim+,and CD34% can predicting the CFU-C as a qucik facter. After cyropreserved, the mature granulocyte were inpaired than lymphocytes,and the segments attaching to the bag gave the equal result with the bag. And to assay the viable cells ,7aad wes the better method than trypan blue method.

Poster Board Number: F-2100

NANOLITER DROPLET VITRIFICATION FOR BLOOD BANKING

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Introduction Blood banking has a broad public health impact influencing millions of lives daily, particularly during military conflicts and natural disasters. Long-term storage techniques of Red Blood Cells (RBCs) can revolutionize how blood is handled in war, prevent wastage and reduce shortages as well as costs. Blood banking could potentially benefit from the advantages of vitrification. However, this has not been incorporated into transfusion medicine mainly due to throughput challenges. The aim of this study is to develop and evaluate an automated high throughput vitrification method utilizing cell encapsulating droplets. This could potentially overcome some of the limitations by lowering the required cryoprotectant agent (CPA) concentrations and achieving ultra-rapid cooling rates via vitrifying RBCs encapsulated in small droplet volumes. We also evaluated naturally occurring non-toxic agents, i.e., Ectoin, to vitrify nanoliter droplets. Materials and Methods Nanoliter droplets were generated from the co-flow stream of CPA loaded

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RBC solution and nitrogen gas flowed through an ejector developed in our laboratory. Following isolation of RBCs, gradual addition of CPA was performed to achieve a final glycerol concentration of 2.5M in a CPA loaded RBC solution (1:1,v/v). 80µl of the solution was ejected onto a collection film. Then, the film was fully submerged into liquid nitrogen to allow vitrification. Once vitrified, the film was dipped into a pre-warmed (37°C) thawing medium. The mixture containing RBCs and 2.5M glycerol is then collected and centrifuged at 2000 g for 10 minutes at 20°C. Following centrifugation, the supernatant was retained and tested for hemolysis index (H-index) and Lactate dehydrogenase (LDH) concentrations. The results were compared to the control where the RBC-CPA mixture was pipetted directly into pre-warmed thawing solution. Results In the existing RBC vitrification study, droplet volumes smaller than 0.25nL reduced potential damage by using relatively low CPA levels allowing, rapid cooling and warming rates. This significant volume reduction allowed RBCs to be vitrified using CPA concentrations as low as 2.5M. Following each step in the vitrification procedure, H-index and LDH levels were evaluated. No significant hemolysis was observed. Additionally, as a proof-of-concept, we vitrified droplets of cryo-medium containing 9%v/v of Ectoin. Conclusions We developed and evaluated the nanoliter droplet cryopreservation method for scalable vitrification of blood by generating nanoliter droplets that were vitrified and thawed at low CPA levels. This approach has the potential to improve the efficiency of global blood inventories and enable new technologies in the field of transfusion medicine.

Poster Board Number: F-2101

UMBILICAL CORD BLOOD STEM CELL TRANSPLANT: AN EXPERIENCE FROM INDIA

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Human cord and placental blood provides a rich source of hematopoietic stem cells (HSCs). Hence umbilical cord blood stem cells have been used to reconstitute hematopoiesis in children suffering from malignant and non malignant diseases, post myeloblative dosage of chemo-radiotherapy. Although there have been reports of post-engraftment transplant rejection, early results confirm cord blood as an adequate HSC source for short and long term engraftment. In fact, the incidence and severity of graft versus host disease has been low even in HLA mismatched transplants. We hereby report about 11 cord blood transplant cases (age range 4 to 18 years, median 9 years) in Eastern India. The case distributions were as follows: Thalassemia 6, Aplastic Anaemia 3, Acute Myeloid Leukemia 1 and Diamond-Blackfan Anaemia 1. Six were males and 5 females. Thalassemia and leukemia patients received myeloablative protocol (Busulfan, Cyclophosphamide), whereas patients suffering from Aplastic Anaemia and Diamond Blackfan syndrome received immunosuppressive protocol (Antithymocyte Glubulin, Fludarabine and Cyclophosphamide). Graft was collected from 7 siblings [4 HLA identical (6/6); 3 unrelated matches (6/6)] and 1 unrelated mismatched donor (3/6). Engraftment was observed in 10 of 11 (90.9%) cases. Our sample size of 11 patients had median recipient weight of 25 kg. Median number of nucleated cells was 4.3 x 107/kg (range 2.1-16 x 107/kg) and median number of CD34 was 3.9 x 106/kg. Median recovery time was 24 days for neutrophils, 48 days for platelets, 68 for hemoglobin. In the past 11 years (08/2000-08/2011), which included a 3 years sabbatical for technical upgrading, we have performed 11 cord blood transplants with 36.3% disease free survival cases and a median follow-up period of 4.6 years. Out of 6 thalassemia patients, 1 did not engraft and 4 rejected the graft 3-6 month post transplant. Only 1 case, where we used a combination of bone marrow and cord blood, had durable engraftment. There was a significant association between non engraftment/rejection and the number of cells infused but no association was found with HLA mismatch. Although in cases of Aplastic Anaemia and non malignant conditions, cord blood (both related and unrelated) proved to be an effective stem cell source. In case of Thalassemia patients, bone marrow continues to be the optimal stem cell source in preference to cord blood, which was proved through our experience, to be an inferior resource.

Poster Board Number: F-2102

HUMAN ESC/IPSC-DERIVED MESENCHYMAL STROMA CAN SUPPORT HEMATOPOIETIC PROGENITORS

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Hematopoietic cells *in vivo* are surrounded by mesenchymal stroma. And the impairments in their interactions are thought to be a cause of some hematological defects. The in vitro differentiation of human embryonic stem (ES) and induced pluripotent stem (iPS) cells is a tool to understand the *in vivo* mechanisms of various cell development and behavior. In hematology, ESC/iPSCs are also a feasible cell source to investigate hematological defects. Despite the recent establishment of several methods to generate ESC/iPSC-derived blood cells, however, the role of mesenchymal stroma in hematopoietic cell development, survival and specification is still unclear. Here we report the human ESC/iPSC-derived hematopoietic stroma capable of efficiently supporting hematopoietic cell survival and lineage commitment in vitro. We manually lifted non-hematopoietic cells from our previously reported hematopoietic differentiation culture, and expanded them in serum-free culture condition. After five to ten passages, they expressed mesenchymal stroma-like surface markers such as CD73, CD29 and CD105. Methylcellulose colony forming assay indicated that they can support cord blood hematopoietic stem cells and generate immature myeloid and erythroid progenitors as long as a week. They are expected to elucidate the detail mechanisms underlying the survival and commitment of normal hematopoetic cells.

Poster Board Number: F-2103

THE MDS1-EVI1 REGULATES HEMATOPOIETIC STEM CELL DORMANCY IN THE MOUSE

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The quiescence of hematopoietic stem cells (HSC) is essential to ensure an adequate supply of blood cells throughout life. Unregulated proliferation of stem cells can lead to premature exhaustion and an inability to respond to stress. *Mds1* represent an alternative start of transcription for the myeloid stem cell regulator *Evi1*, and generate a fusion transcript of Mds1-Evi1 (ME) that encode a 150kd protein of unidentified function. To investigate the role of ME in adult hematopoiesis, we created a LacZ knock-in allele of *Mds1* in the mice. Unlike Evi1 knockout mice, homozygous ME deficient mice are viable and fertile but are small in size. Bata-gal marking studies revealed that within hematopoietic cells, ME is exclusively expressed in Lin-/c-kit+/Sca1+ (LKS) cells. The number of long term repopulating cells (CD150+CD48- LKS) is greatly reduced in Mds1 knockout mice compared to that of wild type. Furthermore, HSC

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in *Mds1* knockout mice has a higher proliferation rate, the number of GMP is dramatically increased in knockout mice. With aging, hematopoietic marrow shows focal aplasia in caudal vertebrae. When stressed with injection with myelotoxic 5-FU, ME-deficient mice fail to recover. These features point to a key role of ME in regulating the balance between quiescence and renewal of hematopoietic stem cells, particularly in the setting of stress.

Poster Board Number: F-2104

P27^{KIP1} MAINTAINS A SUBSET OF LEUKEMIC STEM CELLS IN THE QUIESCENT STATE IN MURINE MLL-LEUKEMIA

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MLL (mixed-lineage leukemia)-fusion genes induce the development of leukemia through deregulation of normal MLL target genes, such as HoxA9 and Meis1. Both HoxA9 and Meis1 are required for MLL-fusion gene-induced leukemogenesis. Co-expression of *HoxA9* and *Meis1* induces acute myeloid leukemia (AML) similar to that seen in mice in which MLL-fusion genes are over-expressed. p27^{kip1} (p27 hereafter), a negative regulator of the cell cycle, has also been defined as an MLL target, the expression of which is up-regulated in MLL-leukemic cells (LCs). To investigate whether p27 plays a role in the pathogenesis of MLL-leukemia, we examined the effects of *p27* deletion (*p27*$^{-/-}$) on MLL-AF9 (MA9)-induced murine AML development. HoxA9/Meis1 (H/M)-induced *p27* wild-type (*p27*$^{+/+}$) and *p27*$^{-/-}$ AML were studied in parallel as controls. We found that LCs from both MA9-AML and H/M-AML can be separated into three fractions, a CD117^{-}CD11b^{hi} differentiated fraction as well as CD117^{+}CD11b^{hi} and CD117^{+}CD11b^{lo}, two undifferentiated fractions. The CD117^{+}CD11b^{lo} fraction, comprising only of 1-3% of total LCs, expresses higher levels of early hematopoietic progenitor markers but lower levels of mature hematopoietic cell markers compared to other populations of LCs. p27 is expressed and is required for maintaining the quiescent and drug-resistant states of the CD117^{+}CD11b^{lo} fraction of MA9-LCs but not of H/M-LCs. *p27* deletion significantly compromises the leukemogenic capacity of CD117^{+}CD11b^{lo} MA9-LCs by reducing the frequency of leukemic stem cells (LSCs) but does not do so in H/M-LCs. In addition, we found that p27 is highly expressed and required for cell cycle arrest in the CD117^{-}CD11b^{hi} fraction in both types of LCs. Furthermore, we found that *c-Myc* expression is required for maintaining LCs in an undifferentiated state independently of proliferation. We concluded that p27 represses the proliferation of LCs, which is specifically required for maintaining the quiescent and drug-resistant states of a small subset of MA9-LSCs in collaboration with the differentiation blockage function of c-Myc.

Poster Board Number: F-2106

A NEW APPROACH FOR ACTIVATING MOUSE HEMATOPOIETIC STEM AND PROGENITOR CELLS BY MANIPULATING GEMININ EXPRESSION

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Polycomb-group (PcG) complex 1 is a multimetric nuclear protein complex, which is known to maintain the repressive states of transcription through monoubiquitination of histone H2A. Mice deficient in either Rae28 or Bmi1, a constituent of PcG complex 1, indicated a crucial role of PcG complex 1 for sustaining the activity of hematopoietic stem cells (HSCs). However, the molecular role for PcG complex 1 in HSC regulation has not been fully understood, because derepression of the Ink4a locus encoding p16INK4a and p19ARF was observed in Bmi1-deficient mice but not Rae28-deficient mice. Hoxb4 is, on the other hand, well known to efficiently induce HSC expansion in vivo and *ex vivo*, and is also a factor which can induce development of HSCs from ES or iPS cells *ex vivo*. Hoxb4 has a DNA binding domain designated as a homeodomain and is so far believed to act solely as a transcription regulator. Genome-wide analyses have been done to identify the downstream target genes responsible for the HSC regulation. The molecular mechanism underlying the Hoxb4-mediated HSC activation, however, still remains unclear. We previously uncovered that either of PcG complex 1 or Hoxb4, a major intrinsic factor for sustaining the HSC activity, directly binds Geminin, a regulator of DNA replication licensing and cellular differentiation (Nature 2004). We further clarified that PcG complex 1 acts as an E3 ubiquitin ligase for Geminin and that accumulated Geminin-protein gave rise to HSC defect in Rae28-deficient mice (PNAS 2008). Curiously, although Hox expression was not affected in Rae28-deficient hematopoietic cells, we found that retroviral transduction of Hoxb4 genetically compensated for HSC defect in Rae28-deficient mice, and demonstrated that Hoxb4 also composes an E3 ubiquitin ligase complex with a Cul4a-Ddb1-Roc1 ubiquitin ligase core component to regulate the protein stability of Geminin, i.e., Hoxb4 transduction reduced accumulated Geminin to recover the HSC activity in Rae28-deficient mice. (PNAS 2010). We further revealed that Hoxa9, a key factor for supporting HSCs and leukemia stem cells, also acts as an E3 ubiquitin ligase for Geminin with Cul4a-Ddb1-Roc1 (manuscript submitted). A series of these findings suggest that Geminin plays a central role in sustaining the activity of HSCs. To further and directly clarify the role for Geminin in hematopoietic regulation, we devised the system for up- or down-regulating Geminin expression in hematopoietic cells. Retroviral transduction of Geminin abrogated the HSC activity, while knock-down of Geminin by the shRNA provided cells with higher clonogenic activity and long term-repopulating ability, indicating that the level of Geminin expression is pivotal for regulating the activity of hematopoietic stem and progenitor cells. Since Geminin expression is higher in the HSC subpopulation and is down-regulated in the progenitors, a role for Geminin in HSC self-renewal attends our particular attention. To further elucidate the molecular role for Geminin in HSCs, we have recently generated Geminin-EYFP knock-in mice to visualize Geminin expression. In this meeting, we argue for a role for dynamic expression profile of Geminin in the hematopoietic system and for a possible implication in developing a new technology for expanding HSCs *ex vivo*.

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Poster Board Number: F-2107

SOX-F FAMILY PROTEINS HAVE ROLES IN THE MAINTAINANCE OF IMMATURE PHENOTYPE OF THE HEMATOPOIETIC CELL CLUSTERS IN THE AORTA-GONAD-MESONEPHROS REGION OF MOUSE EMBRYOS

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In the aorta-gonad-mesonephros (AGM) region, from which definitive hematopoiesis firstly arises in the mouse embryo during development, the hematopoietic cell clusters are found in the endothelium of the dorsal aorta. By the analysis of transplantation into irradiated mice, the hematopoietic cell clusters contain the hematopoietic stem/progenitor cells. Sry-related high mobility group box (Sox) 17 is a transcriptional factor known as a marker of endodermal cells. It was recently reported that Sox17 conditional knockout mice displayed a decrease in the number of hematopoietic stem cells in the fetal and neonatal, but not the adult stage. In the present study, we examined the function of Sox17 and related molecules in the same subfamily (SoxF subfamily), i.e. Sox7 and Sox18, in the hematopoiesis of the AGM region. SoxF proteins were expressed in endothelial cells lining dorsal aorta and the hematopoietic cell clusters of embryonic day (E)10.5 mouse embryo. Overexpression of SoxF proteins into E10.5 CD45lowc-Kithigh AGM cells, which are components of the hematopoietic cell clusters in the AGM region and show the high hematopoietic activity, led to form cell clusters with a ball-like structure even under the differentiation-inducing culture conditions with stromal cells. These cluster cells maintained the immature morphology and had a high ability to form hematopoietic colonies *in vitro*. The Sox17-induced cluster cells expressed transcriptional factors that are essential for mouse fetal hematopoiesis (GATA-2, c-Myb, and AML1) as well as endothelial cell markers (CD31 and VE-cad), and were found to be derived from a single cell. Appearance of the cell clusters in the coculture of CD45lowc-Kithigh AGM cells with the stromal cells was not observed by overexpression of other Sox family proteins, Sox9 and Sry. By the conditional shutdown of the Sox17 gene in Sox17-expressing ball-like cell clusters, hematopoietic cells emerged from the ball-like cell clusters and then differentiated on the stromal cells. After long-term cultures with stromal cells, the Sox7 and Sox17-expressing AGM cells were capable of maintaining the ability of reproducing ball-like cell clusters with mix-lineage colony forming ability in semi-solid media. Moreover, transplantation of Sox17-expressing cells to the recipient mice resulted in the long-term repopulation of myeloid cells, erythroid cells, and T-lymphocytes, but repopulation of B-lymphocytes was very faint. Knockdown of Sox17 and Sox7 in Sox18-deficient CD45lowc-Kithigh cells by short hairpin RNA led to reduction of colony-forming activity in semi-solid media. These results suggested that SoxF family proteins, especially Sox17, contribute to maintenance of the undifferentiated state of the hematopoietic cell clusters in the AGM region.

Poster Board Number: F-2108

IN VIVO EXPANSION OF MOUSE HEMATOPOIETIC STEM CELLS BY PURIFIED RECOMBINANT HUMAN TAT-HOXB4 PROTEIN

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Bone marrow transplantation after chemotherapy or radiation therapy requires sufficient number of hematopoietic stem cells (HSCs) to ensure success. Recombinant human homeobox B4 (HOXB4) protein was recently demonstrated to effectively expand human bone marrow HSCs in vitro. We previously demonstrated that TAT-HOXB4 could also expand cord blood and peripheral blood HSCs in vitro. However, in vitro usage of TAT-HOXB4 requires purification of HSCs followed by in vitro expansion, which may not be feasible for different kinds of patients. The ideal approach will be to directly expand HSCs *in vivo*, which will require large amounts of purified recombinant TAT-HOXB4. Due to the extreme instability of HOXB4, it remains a big hurdle to obtain enough amount of TAT-HOXB4 to be used *in vivo*. To achieve this goal, we mass-produced and purified the recombinant TAT-HOXB4 protein to clinical scale and administered the protein *in vivo*. Balb/c mice were used as hosts to test the ability of recombinant TAT-HOXB4 protein to expand mouse HSCs. Recombinant TAT-HOXB4 proteins at the dosage of 100 μg/kg were injected into tail vein 4 times per day for 5 days. The results showed that the number of total mouse HSCs (measured by CD34+ staining cells) increased approximately three fold compare to the control-treated animal. Further experiments using the semisolid cloning assay, human long-term culture-initiating cells (LTC-ICs) assay and nonobese diabetic-severe combined immunodeficiency (NOD-SCID) mice repopulating assay all showed that TAT-HOXB4 expanded HSCs while retaining their repopulating capacity and multipotency. In addition, recombinant TAT-HOXB4 protein enhanced engraftment of bone marrow transplants, improved bone marrow re-population and increased the number of circulating blood cells in mice treated with prior chemotherapy or irradiation. All these results demonstrated that recombinant TAT-HOXB4 protein expanded mouse HSCs *in vivo*, which served as a basis for the potential usage of TAT-HOXB4 in clinical application.

Poster Board Number: F-2109

PU.1 IS REQUIRED FOR THE DEVELOPMENT OF CLP IN MOUSE ADULT HAEMATOPOIESIS

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PU.1, often referred as Spi1 in humans, is an ETS-family transcription factor (TF) that is widely expressed in haematopoietic cells. Through the control of the expression of several vital cytokines receptors, and its interaction with a number of key TFs, PU.1 is part of the complex transcriptional network that ensures that a single haematopoietic stem cell (HSC), can develop into different precursor populations such as lymphoid primed multipotent progenitors (LMPP), common myeloid progenitors (CMP) and common lymphoid progenitors (CLP), and eventually differentiate into multiple blood lineages. PU.1 has long been known to be essential for fetal haematopoiesis. More recently we have developed a conditional allele of PU.1 and demonstrated that PU.1 is also required for the differentiation of adult bone marrow HSCs in the lymphoid and myeloid lineages. However, there is a caveat to determining at which developmental stage PU.1 is required, as many of the cytokine

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receptors and markers used to identify different precursor populations are direct targets of PU.1. For example, interleukin (IL)7Rα which is used to identify CLPs, and the receptor tyrosine kinase FLT3, which is important in identifying LMPPs, are both PU.1 targets. We have now overcome this limitation by crossing mice with the conditional PU.1 allele, with mice bearing Recombinase Activation Gene (RAG)1-GFP reporter that marks the early lymphoid progenitors. Using this system, we are able to identify a lymphoid precursor population from PU.1-deficient bone marrow corresponding to LMPP in the absence of FLT3 expression, whereas the downstream CLP population was absent. This finding shows that the deletion of PU.1 in adult mice results in a block in the LMPP stage, and prevents further differentiation to the CLP and mature lymphocytes. We are now further dissecting the role of PU.1 specifically in LMPP with the aid of RAG1, and also in the developmental stage between LMPP and CLP. PU.1 may be required for the upregulation of some cytokine receptors so that LMPP can develop normally into CLP. To further investigate this question, we are currently performing gene expression profiling on PU.1 progenitors, to determine other targets of PU.1.

Poster Board Number: F-2110

CHARACTERIZATION OF MAST CELLS GENERATED BY DIFFERENTIATION FROM MOUSE INDUCED PLURIPOTENT STEM CELLS

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Mast cells are derived from bone marrow-derived hematopoietic progenitor cells that migrate into all vascularized tissues where they complete their maturation. Rodent mast cells are generally classified into two phenotypically distinct populations: connective tissue-type mast cells (CTMCs) and mucosal-type mast cells (MMCs). Because recent studies have demonstrated that CTMCs are involved in a wide variety of immune responses, it is needed to establish *in vitro* culture system of CTMCs. Differentiation of hematopoietic cells from ES or iPS cells using OP9 cells as feeder cells has been reported, including erythrocytes and B lymphocytes. Here, we report that iPS cells co-cultured with OP9 cells differentiated into mast cells, and we characterized them using morphological and functional assays. May-Grunwald-Giemsa and acidic toluidine blue staining showed no differences between conventional mouse bone marrow-derived mast cells (BMMCs) and iPS cell-derived mast cells (iPSMCs). While the majority of BMMCs are known to be Alcian blue-positive and Safranin O-negative, iPSMCs were positive for both Alcian blue and Safranin O staining. iPSMCs showed a more elevated tryptase and carboxypeptidase A activity, as compared with that in BMMCs. Moreover, β-hexosaminidase release by cationic secretagogues as a degranulation index in iPSMCs was markedly elevated in comparison with that in BMMCs, suggesting that iPSMCs were more matured than BMMCs. Electron microscopic analysis of mast cells revealed that iPSMCs contained more granules than BMMCs. Maturation of iPSMCs was co-cultured with Swiss 3T3 fibroblasts in the presence of SCF, could be facilitated toward a CTMC-like phenotype, however it remains unclear what factors are involved in the maturation of iPSMCs. To investigate whether maturation of mast cells requires physical contact between mast cells and feeder cells, mast cells were co-cultured with feeder cells in the Transwell apparatus, demonstrating that both soluble factors and cell-adhesion molecules, which were derived from feeder cells, played an important role in mast cell maturation. Thus, iPSMCs have many distinct characteristics from BMMCs and can be used as an *in vitro* model of CTMCs to further investigate their functions.

Poster Board Number: F-2111

ENDOGENOUS EXPRESSION LEVELS OF POLYCOMB CBX FAMILY MEMBERS IN MOUSE HEMATOPOIETIC STEM AND PROGENITOR CELLS

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Hematopoietic stem cells (HSCs) are multipotent cells able to undergo either self-renewal or differentiation and the specific balance of these cell fate decisions is essential to sustain proper blood cell production. Polycomb Group proteins (PcG) were shown to be implicated in maintaining this balance by repressing genes involved in proliferation or differentiation. PcG proteins reside in two multi-protein complexes: the Polycomb Repressive Complex 1 and 2 (PRC1 and PRC2). PRC2 introduces chromatin repressive marks (H3K9/27me3) which are read by PRC1 that subsequently alters higher chromatin organization. PRC1 consists of four core subunits, of which Cbx family members recognize and catalyze PRC1 binding to H3K9/27me3. During evolution, the number of genes encoding for every PcG subunit has expanded. Therefore, in mammals four Cbx family members (Cbx2, Cbx4, Cbx7 and Cbx8) exist that can integrate within PRC1 . However, the reason for this Polycomb diversification and how different Cbx orthologs contribute to PRC1 function is unclear. To study the function of distinct Cbx-containing PRC1 subcomplexes in hematopoietic stem cells, we first investigated the pattern of Cbx abundance along hematopoietic stem cell differentiation. To this end, we subfractionated bone marrow cells by fluorescent activated cell sorting (FACS) in long-term hematopoietic stem cells (LT-HSC; $Lin^-Sca\text{-}1^+c\text{-}Kit^+CD48^-CD34^-CD150^+$), short-term hematopoietic stem cells (ST-HSC; $Lin^-Sca\text{-}1^+c\text{-}Kit^+CD48^-CD34^+CD150^-$), multipotent progenitors (MPP; $Lin^-Sca\text{-}1^+c\text{-}Kit^+CD48^+CD34^+CD150^-$), committed lymphoid progenitors (CLP; $Lin^-Sca\text{-}1^{mid}c\text{-}Kit^{mid}CD127^+$), committed myeloid progenitors (CMP; $Lin^-Sca\text{-}1^-c\text{-}Kit^+CD127^-CD34^{mid}CD16/32^{mid}$), granulocyte/monocyte progenitors (GMP; $Lin^-Sca\text{-}1^-c\text{-}Kit^+CD127^-CD34^{high}CD16/32^{high}$), megakaryocyte/erythrocyte progenitors (MEP; $Lin^-Sca\text{-}1^-c\text{-}Kit^+CD127^-CD34^{low}CD16/32^{low}$), and mature myeloid cells ($Gr\text{-}1^+$). Quantitative rt-PCR showed that Cbx7 expression gradually decreased along hematopoietic stem cell differentiation, while the expression of Cbx4 increased. Expression of Cbx2 seemed to be specific for CLPs and Cbx8 was found to be equally expressed in all analyzed populations. In addition, Cbx7 transcript levels showed to be the highest among all Cbx orthologs in LT-HSCs. Analysis at the protein level was done in sorted LSK ($Lin^-Sca\text{-}1^+c\text{-}Kit^+$), progenitors ($Lin^-Sca\text{-}1^-c\text{-}Kit^+$) and mature myeloid cells ($Gr\text{-}1^+$) and confirmed the decreasing pattern of Cbx7 expression upon differentiation. Cbx2 and Cbx8 proteins were detectable in progenitors and mature myeloid cells, however this did not correlate with the mRNA levels. Although Cbx8 was not shown to be upregulated at the transcriptional level along HSC differentiation, the analysis at the protein level showed that there was a higher abundancy of Cbx8 in differentiated compared to immature cells. In summary, Cbx expression analysis showed transition from Cbx7 predominance in hematopoietic stem cells, to Cbx2/Cbx4 predominance in committed progenitors, followed by Cbx4/Cbx8 predominance in more differentiated hematopoietic cells. These Cbx expression data suggests an exclusive role for Cbx7-containing PRC1 complexes in the most primitive hematopoietic compartment, while other

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Cbx orthologs might function in government of the differentiation program.

Poster Board Number: F-2112

THE MOLECULAR NETWORK INVOLVED IN ASYMMETRIC CELL DIVISION OF MOUSE HEMATOPOIETIC STEM CELLS.

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Asymmetric cell division (ACD) is a mechanism by which the defining hematopoietic stem cell (HSC) properties of self-renewal and multi-potentiality can be propagated for the entirety of a lifespan. Studies in model invertebrate systems has provided knowledge of the molecular interactions governing ACD and suggests the combination of polarisation of cell fate determinants together with the cytoskeletal networks governing mitotic spindle during cell division as pivotal cell intrinsic components of the ACD process. With the aim of identifying the molecular network important for ACD within the mammalian haematopoiesis context of self-renewal, we performed an in vitro expansion to *in vivo* transplantation screen of candidate cell polarity genes to assess for HSC self-renewal. Results of this screen as recently published (Ting SB et al. Blood 2011 Dec14 Epub), identified six out of 43 polarity genes were able to provide a HSC reconstitution benefit. One of these 6 candidates, Ap2a2 is a potential cell fate determinant as it is a known binding partner of the established cell fate determinant, Numb and we have shown AP2A2 segregates asymmetrically during HSC mitotic divisions. Interestingly, four of the other candidates are either direct (Tmod1, Gpsm2, Kif3a) or indirect (Racgap1) components of the cytoskeletal networks. These results within the haematopoietic system thereby mirroring the two main networks involved in invertebrate ACD processes. The sixth candidate (Ccnb1) is a cell cycle related gene. As AP2A2 is part of the AP2 heterotetramer complex pivotal for clathrin-coated endocytosis of ligand-bound intramembranous receptors, we have performed immunofluorescent co-staining of AP2A2 with established markers of the endocytosis-lysosome network in both purified mouse bone marrow Lin-Sca+Kit+ (LSK) HSC and leukemia cell line populations, and analysed these under N-SIM super resolution microscopy. The presence or absence of co-localisation of AP2A2 with these cytoplasmic vesicular components will be reported. To assess the functional consequence of perturbation of Ap2a2 we have used overexpression and shRNA knockdown of Ap2a2 in the LSK population, with read-outs being a transferrin receptor internalization assay, together with flow cytometry and confocal measurement of IL3, IL6, SCF and Tpo cytokine receptors, all of which are not only integral to HSC signaling but are also internalized via AP2-clathrin mediated endocytosis. To further investigate the role of the cytoskeleton in HSC self-renewal and whether alteration in mitotic spindle orientation during HSC mitosis may be deterministic for HSC fate, we have utilised a combination of confocal microscopy of established cell fate determinant markers and real-time videomicroscopy to assay for AP2A2 symmetrical or asymmetrical localisation during HSC divisions during perturbation of the relevant cytoskeletal candidate genes.

Poster Board Number: F-2113

MARROW REGENERATION- MOUSE HSCS TURNOVER AND MICRO-ENVIRONMENTAL CUES

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Adult stem cells hold many promises in regenerative medicine. The hematopoietic stem cell (HSC) is the best-characterized somatic stem cell so far, but *in vitro* expansion has been unsuccessful, limiting the future therapeutic potential of these cultured cells. HSC niche plays a key role in its expansion. Niches are local tissue microenvironments to maintain and regulate stem cells. There are two types of HSC niche: Osteoblastic niche which maintains stem cells in quiescent state and Vascular Niche which activates the stem cell for proliferation. However, to maintain stem-cell homeostasis, the niche not only provides a shelter to protect the stem cell from any physiological stress or challenges but also restrains it from differentiation (which could exhaust the stem-cell pool) and over-production (which could result in tumor formation). The objective of this study was to find out donor stem cells renewal, location in marrow niche, cell cycle status and to identify the system of marrow derived novel factors that may have control on hematopoiesis. We performed experiments using mouse as a model for the study. We took C57 BL/6J mouse expressing alloantigen as donor and recipient mice (to avoid immunological rejection and to identify donor cells). Bone marrow cells were harvested from the donor mice and transplanted via tail vein into the sublethally irradiated recipient mice. Time dependent increase in donor stem cell number was seen. Observations shows that there is an increase in donor absolute stem cell population upto 15 day post transplantation and then it maintains a plateau. We further confirmed this experiment by competitive marrow repopulation assay where we found a dose dependent increase in absolute stem cell population. Cycling status of the cells was determined post transplantation using Hoechst and PyroninY staining of HSCs at different time points. It was found that percentage of donor HSCs at GO phase decreased when compared to the cells at G1 and SG2M phase. In case of the recipient, the absolute stem cell population was higher in the quiescent stage i.e. the Go phase. Immunohistochemical analysis further confirmed the colonization and localization of donor HSCs in recipient mice. Global gene expression analysis was performed with marrow stromal cells isolated at different time points after BMC transplantation in an irradiated recipient to identify the key molecules/genes in HSC division and expansion. We identified many genes of the stromal compartment which was up regulated and also down regulated with time. The present study demonstrates the importance of various key molecules in the HSC niche, which may help in the maintenance and differentiation of HSCs in the bone marrow. Further validation of these genes through knockdown and over-expression studies is under progress to establish their importance in HSC maintenance and proliferation.

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MOBILIZATION OF MOUSE HEMATOPOIETIC STEM/PROGENITOR CELLS BY VASCULAR ENDOTHELIAL GROWTH FACTOR IS MEDIATED BY REDUCTION OF MESENCHYMAL STROMAL CELLS IN BONE MARROW

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Hematopoietic stem/progenitor cells (HSPCs) exist within the bone marrow (BM) in a steady state, while they are mobilized to circulation following administration of granulocyte-colony stimulating factor (G-CSF). Several studies have demonstrated that HSC mobilization by G-CSF is mainly mediated by disruption of CXCL12/CXCR4 signals. As like the case with G-CSF, vascular endothelial growth factor (VEGF) is reported to have a potent HSPC mobilization activity. However, the detailed mechanisms of HSC mobilization by VEGF treatment have not been examined. To clarify the effect of VEGF on the BM cell mobilization and BM environment, we investigated the number of hematopoietic progenitor cells and the expression level of cytokines and adhesion molecules, both of which are involved in HSPC retention in BM, after the intravenous injection of VEGF-expressing adenovirus (Ad) vector (Ad-VEGF) into mice. We found a significant increase in the number of hematopoietic progenitor cells with the colony-forming potentials in peripheral blood, while the number of progenitor cells in BM was decreased in Ad-VEGF-injected mice. This indicates that plasma elevation of VEGF leads to the mobilization of hematopoietic progenitor cells to circulation. We also found that, unlike G-CSF, mobilization by VEGF was unrelated with CXCL12/CXCR4 signals, because the amount of CXCL12 in BM did not change after Ad-VEGF administration. On the other hand, as like the G-CSF, the expression levels of vascular cell adhesion molecule-1 (VCAM-1) and angiopoietin-1(Ang-1) in BM were decreased in Ad-VEGF-injected mice. Thus, these results suggest that VEGF alters the BM environment by different pathway to G-CSF. We next measured the number of mesenchymal stromal cells, which are recently reported as niche cells to support HSPCs, in BM following Ad-VEGF administration. Flow cytometric analysis and CFU-F assay revealed the reduction of mesenchymal stromal cells in BM in Ad-VEGF-injected mice. Attachment of donor cells in BM after BM transplantation was also reduced in mice injected with Ad-VEGF, indicative of a decrease in niche cells. Furthermore, we observed a dose-dependent chemoattractive effect of VEGF on mesenchymal stromal cells *in vitro*. These data suggest that overexpression of VEGF alter the distribution of mesenhymal stromal cells in BM, and that mesenchymal stromal cells might be also mobilized to peripheral tissues. Taken together, our results imply that VEGF overexpression in mice changes not only the gene expression level of cytokine and adhesion molecules, except CXCL12/CXCR4 signals, but it also changes the number of mesenchymal stromal cells in BM. The decreased number of mesenchymal stromal cells in BM by VEGF would then lead to HSPC egress from BM.

Poster Board Number: F-2115

AGED LNK-DEFICIENT HEMATOPOIETIC MOUSE STEM CELLS SHOW ENHANCED SELF-RENEWAL WITHOUT PREMATURE EXHAUSTION

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Blood cells are continuously produced from hematopoietic stem cells (HSCs) within the bone marrow (BM). A major property of stem cells is their ability for self-renewal, which is important to maintain the HSC pool for the lifespan of the organism. However with aging, the HSC compartment undergoes distinct changes in terms of clonal repopulating ability and myeloid skewing of differentiation potential. Thus aging is characterized by anemia, decreased lymphocytes production, leading to impaired immune response, and increased production of cells from myeloid lineage, which results in an increased incidence of myeloproliferative neoplasms (MPN). Thus in spite of an age-related increase in the number of HSCs in old mice, the repopulating and self-renewal ability are reduced. Studies of HSCs from older mice presented us with a model to investigate the role of the adaptor protein Lnk in age-related HSC functional decline. Lnk is an important negative regulator of HSC homeostasis and self-renewal. By adulthood (at two-month of age), Lnk-/- mice have a 10-fold increase in HSC numbers and superior multi-lineage repopulation after bone marrow transplantation (BMT). Here we report that the expansion of long-term repopulating HSCs, both phenotypically and functionally, is also evident in old Lnk-/- mice. Importantly, HSCs from aged Lnk-/- mice show enhanced self-renewal without premature exhaustion, as evidenced by serial transplants of either unfractionated or purified HSCs. Using colony-forming assays we found that Lnk-/- mice show expansions in progenitor numbers and particularly myeloid progenitors. This is in agreement with our previous report that Lnk-/- mice spontaneously develop a chronic myelogenous leukemia-like MPN. These studies in mice also correlate well with recent reports from patients with MPN, where Lnk loss-of-function mutations have been found. Furthermore, studies in the signaling mechanisms by which Lnk affects HSC aging may provide new insights into stem cell aging and facilitate clinical applications to stem cell therapy in the treatment of aging- related diseases. At the same time, these studies support the downside concerns with improvement of aged HSC function of increased risk of MPNs and leukemic states. Ongoing studies in the laboratory using young and old wildtype and Lnk deficient HSCs should shed additional light on the mechanism(s) underlying the changes in HSCs number and function during aging, and determine the role of Lnk in these interactive processes.

Poster Board Number: F-2116

EXPANSION OF UMBILICAL CORD BLOOD DERIVED CD133 STEM/PROGENITOR CELLS ON BIOCOMPATIBLE MICROWELLS

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Hematopoietic stem cell transplantation (HSCT) is a theraputic approach in treatment of hematological malignancies and incompatibility of Bone marrow . Umbilical cord blood (UCB) known as an alternative for hematopoietic stem / progenitor cells (HPSC) for in allogenic transplantation . The main hinderance in application of HPSC derived from umbilical cord blood is the low volume of collected samples . So, *ex vivo* expansion of HPSCs is the useful ap-

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proach to overcome this restriction . The goal of using this systems is to produce appropriate amount of hematopoietic stem cells , which have the ability of transplantation and long term hematopoiesis . The routine system of hematopoietic stem cells expansion is using expansion cytokine coaktail in 2- Dimensional (2D) microenvironment. In this microenvironemt cells only affected by cytokines and there is not any influence of cell - matrix intractions , migration and attachment. But 3- Dimensional (3D) microenvironments mimickes stem cells niche . Synthetic biomaterials such as microwells based on PDMS coated with Extracellular matrix (ECM) proteins is used to produce synthetic niches . These 3D structures , chemical and mechanical properties of these microwells leads to activation of adhesion , proliferation , differentiation and migration of CD133+ cells with highly similarity to ECM . In these microwells analouge materials of ECM or natural proteins such as collagen , fibronectin and laminin modulate the rate of prolifration and expression of genes participated in proliferation. Materials : In current study CD133+ cells were isolated from umbilical cord blood and CD133 cells were isolated by MACS technique . Then isolated cells were seeded on microwells which were prepared by softlitography method . Then isolated cells were seeded on microwells which were prepared by softlitography method . proliferation of cells were analyzed by assassing the expression of CD133 marker and cells were counted on defined time periods on day 7 and 14 . we compared our experiments with routine cell culture conditions Results : we observed that CD133 cells proliferation were significantly increased on PDMS based microwells coated with collagen compared to 2D cell culture conditions. Conclusion : *Ex vivo* expansion of hematopoitic stem cells on 3D conditions mimicks stem cell niche and this condition helps cells to proliferate and migrate similar to the conditions in body with less differentiation compared to 2D cell culture conditions .

Poster Board Number: F-2117

EP4 RECEPTOR-DEPENDENT DIRECT AND -INDEPENDENT INDIRECT EFFECTS OF PROSTAGLANDIN E2 IN THE REGULATION OF ADULT HEMATOPOIETIC STEM/PROGENITOR CELLS

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Regulation of the hematopoietic stem cells/progenitor cells (HSPCs) is depending on the interaction with their niche. Prostaglandin E2 (PGE2) is a mediator of various physiological systems and pathological states. PGE2 signaling through its receptor, EP2 and EP4, regulates HSC development and enhances repopulation activity by the interactions with the Wnt/β-catenin pathway. However, it has not been fully determined which receptor is responsible for PGE2 signaling in adult HSCs. Furthermore, the indirect effects of PGE2 on hematopoiesis through other niche cell compartments have not been well understood. Here we analyzed the function of PGE2 signaling in the regulation of adult bone marrow (BM) HSPCs using EP2 and EP4 receptor KO mice and receptor selective agonists (KO mice were kindly provided by Dr. Shu Narumiya (Kyoto University)). First, the function of EP2 and EP4 receptors in PGE2-mediated direct HSPC regulation was analyzed. Digital PCR analysis revealed that EP2 transcripts were present in much lower numbers than EP4 transcripts in HSPCs. EP2-deficient mice showed normal hematopoiesis. In contrast, EP4-deficient mice demonstrated an alteration in the proportion of differentiated cells (higher percentage of T cells and myeloid cells and a lower percentage of B cells). Knockdown of EP4 reduced long-term reconstitution (LTR) capacity of Lin-Sca-1+c-Kit+ (LSK) cells. Meanwhile, EP4 selective agonist increased the colony formation and enhanced the LTR activity of LSK cells. Immunoblot analysis revealed that PGE2 as well as EP4 agonist treatment increased phosphorylation of GSK3β through a cAMP/PKA pathway. Next, we examined the effect of PGE2/EP4 signaling on the recovery of HSCs after myelosuppression. Mice treated with PGE2 showed a higher frequency of LSK cells at 6 and 8 days post 5-FU treatment. The LSK cells derived from the 5-FU-injected PGE2-treated mice had significantly higher expression of cell cycle-related genes and HSC markers compared to the control. These data indicated that *in vivo* PGE2 treatment accelerates HSPC proliferation after myeloablation and/or protects stem cells from 5-FU-induced apoptosis. Although *in vivo* treatment with EP4 agonist promoted the recovery of HSPCs after 5-FU-induced myelosuppression, the effect was less effective than that of PGE2. Then, to investigate the indirect effects of *in vivo* PGE2 treatment on hematopoiesis, we divided bone endosteal cells into three fractions; ALCAM+Sca-1- mature osteoblasts, ALCAM-Sca-1- immature osteoblasts, and ALCAM-Sca-1+ mesenchymal stem/progenitor cells (MSPCs). We found that ALCAM-Sca-1+ MSPCs showed higher levels of expression of EP1-4 receptors compared to other fractions. In the coculture assay, the pre-treatment of ALCAM-Sca-1+ MSPCs with PGE2 but not EP2 and EP4 agonists significantly increased CFU-C and HPP-CFC of LSK cells after coculture. Interestingly, higher levels of Angiopoietin1 and Hyaluronic acid synthetase 1 (HAS1) gene expression were detected in PGE2-treated ALCAM-Sca-1+ population, compared with control ALCAM-Sca-1+ cells. The results indicate that PGE2-stimulated MSPC niche cells support BM recovery after myelosuppression, in addition to direct effect of PGE2 on HSPCs. Together, these data suggest that PGE2 signaling directly regulates adult HSCs through EP4 receptors while indirectly regulates hematopoiesis through MSPCs by stimulating other receptors.

Poster Board Number: F-2118

MORPHOLOGY AND BIOCHEMICAL DIFFERENCES OF MAST CELLS (NCL-2 CELLS, RBL-2H3 CELLS) ON THE NEW EXTRACELLULAR MATRIX; HONEYCOMB FILM

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Mast cells are found in some tissues of the body, and are well known for immune response and allergic reactions. Mast cells are originated from bone marrow precursors expressing the CD34+ molecule as same as basophiles. Though basophiles migrate from the bone marrow as already mature form, mast cells circulate in an immature form in blood, only maturing once in a tissue site. Immature mast cells settle in the tissues, i.e., skin, bronchi, stomach, mouth, and brain, where mast cells differentiate and determine its precise characteristics. As mast cells can determine their characteristics in each organ, we speculate that mast cells may be sensitive to surrounding microenvironment. Recently, polymeric materials have been reported to be useful for biomaterials in the cell culture-based experiments. We have reported the difference of the cell adhesion and proliferation by using three-dimensional films with highly regular patterns of the polymeric materials "Honeycomb film (HCF)". We fabricated this HCF by applying moist air to spread the polymer solution containing a biodegradable polymer and an amphiphilic polymer. Then we succeeded to inhibit the prolifera-

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tion and functions of the cancer cells only, compared with those of the normal cells, on the HCF as extra-cellular matrix. Culturing mast cells on the HCF, we have examined whether the mast cell morphological and biochemical reactions may be changed, because *in vivo* mast cells are influenced by surrounding environment in the way of differentiations. In this study, we observed mast cell proliferation and morphology by scanning electron microscope observation (SEM), actin filament and adhesion molecule by confocal laser scanning microscope observation (CLSM), histamine (HA) and leukotriene B4 (LTB4) release concentration of spontaneous emission by enzyme immuno assay. We used two types of mast cell lines, NCL-2 (proliferative mouse normal mast cells) and RBL-2H3 (rat basophilic leukemia cells). One week after NCL-2 and RBL-2H3 culture on the polystyrene well microplate (control), cover glass, flat film, 3, 5, and 10um HCF, total number of NCL-2 cells proliferated by culture on HCF compared with that on cover glass and flat film ($p<0.05$). While, the number of RBL-2H3 floating cells decreased and adherent cells increased remarkably culturing on HCF comparing on cover glass and flat film. However, the total number of RBL-2H3 did not change. By the result of SEM, RBL-2H3 cells extended widely more than NCL-2 cells on cover glass and flat film, and then both cells adhered on the 3 and 5 um HCF, and raised cell-division particularly. According to the CLSM observation with stained for vinculin, actin, and DAPI for nuclei, the difference was seen between NCL-2 and RBL-2H3 depending on the pore size of HCF. By the results of concentration of HA and LTB4 release, a difference was seen among NCL-2 and RBL-2H3 cells on control, flat film, cover glass, and each size of HCF. Conclusions: 1) The NCL-2 cell line was used for an experiment after the patent acquisition for the first time. These cells are floating cells basically though RBL-2H3 cells are adhesive. NCL-2 cells may be useful for mast cell research because these are not tumor cells. 2) We examined HCF as a regulator for mast cells to control inflammation and allergy. 3) In spite of the floating property, NCL-2 cells proliferated in response to the structure of HCF. 4) A difference of pattern (size of the hole) of HCF may have some influence to the mast cells.

Poster Board Number: F-2119

IDENTIFICATION OF THE EARLIEST BRANCH POINT FOR MYELO-ERYTHROID DEVELOPMENT IN ADULT HEMATOPOIESIS

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In murine hematopoiesis, hematopoietic stem cells (HSCs) have been identified within the LSK fraction of bone marrow cells, which is defined by the absence of lineage-associated surface markers (Lin) and high expression of Sca-1 and c-Kit. The first commitment step at the myeloid versus lymphoid bifurcation has been proposed outside the LSK fraction, where myeloid or lymphoid lineage-committed progenitors such as common myeloid progenitors (CMP) and common lymphoid progenitors (CLP) are prospectively isolated. However, recently, by utilizing mice harboring a fluorescent reporter for GATA-1 transcription factor, we found that the upregulation of GATA-1 occurs within the LSK population; The GATA-1+ LSK population was capable of generating only myelo-erythroid cells but lacks lymphoid potential, suggesting strongly that the earliest myeloid development occurs at the LSK stage. To isolate the earliest myelo-erythroid LSK progenitors in normal mice without utilizing the GATA-1 reporter system, we conducted expression profiling of GATA-1+ LSK cells by cDNA microarray analyses, and identified a cell-surface antigen (X) specifically expressed in GATA-1+ LSK cells. By using this new surface marker X, we could successfully purify a cell population from normal C57B6 mice which gives rise exclusively to granulocyte-macrophage (GM) and megakaryocyte-erythroid (MegE) colonies, but lacks lymphoid potential. Furthermore, X+LSK cells display very potent GM and erythroid expansion as compared to the original CMPs, suggesting that X+LSK might reside upstream of CMPs. The gene expression analysis of the X+ LSK population at single-cell level revealed that this population expresses GM and MegE-associated genes at a high level, but not lymphoid genes, suggesting that the gene expression pattern clearly reflects its lineage differentiation potential. Thus, the X+ LSK population might represent the earliest stage for myelo-erythroid development, upstream of conventional CMP, in normal mice, which should be a useful tool for investigating the molecular mechanisms for hematopoietic lineage fate decision.

Poster Board Number: F-2120

AGEING-ASSOCIATED INTRINSIC AND EXTRINSIC REGULATION OF HEMATOPOIETIC STEM CELL BEHAVIOR

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Lifelong blood formation is maintained by self-renewing hematopoietic stem cells (HSCs) in bone marrow (BM). We recently have demonstrated that HSCs are at any given time divided into two pools, actively cycling and dormant HSCs, with both comparable long-term repopulating capacity, and that they are reversely switched with fluctuating cell cycling activity over time (Takizawa et al., JEM 2011). It was also found that HSCs with high proliferative history during natural aging or serial transplantation tend to be dormant in a permissive environment. This observation let us hypothesize that cell-intrinsic drive toward dormancy is imprinted on HSCs through extensive division. Here we tackle the questions on how the extrinsic and intrinsic factors determine HSC division and cell fate, and what their molecular signature and relative contribution are in the HSC behaviour. To this end, we employ *in vivo* single HSC divisional tracking with CFSE (5(6)-carboxyfluorescein diacetate N-succinimidyl ester) which allows determination of steady-state divisional history and subsequent isolation and functional test of different divisional classes of HSC-containing fractions (LKS or LKS CD34-). CFSE-labeled young (8-12 week old), aged (>2 year old) and young, previously serial-transplanted LKS were transferred into non-irradiated young or aged recipient. Eight week after tracking, cycling (>5x-divided) or dormant (non-divided) LKS were sorted and transplanted into lethally irradiated recipients for monthly follow-up of donor engraftment and lineage repopulation. Consistent with our previous findings, aged HSCs increased dormancy compared to young HSCs when transferred into young animals, confirming cell-intrinsic regulation of dormancy. While fast-cycling aged HSCs showed balanced lineage repopulation in lethally irradiated recipient as did both fast-cycling and dormant young HSCs, dormant aged HSCs contributed to more myelopoiesis than lymphopoiesis, indicating the link between HSC lineage choice and cell cycle activity, in a cell-intrinsic manner. Similar biology as in aged HSCs was observed in HSCs that went through extensive division through serial transplantation. Furthermore, preliminary results showed that both young and aged HSC proliferated faster in an aged compared to a young environment, suggesting the existence of extrinsic drive for HSC division. To dissect aging-associated intrinsic and extrinsic factors to drive HSC division, we performed antibody based protein arrays with young versus aged BM cell lysates followed by bioinfomatical analysis to narrow down key candidate proteins. Expression levels of some fluid factors and cell surface receptors was significantly altered in aged BM, suggest-

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ing their possible role in HSC activation.Our findings suggest that extensive proliferative history, e.g. during natural aging or chronic inflammation, imprints the intrinsic changes on HSCs to keep them dormant and bias their repopulating potential to myeloid readout. The HSC dormancy can be reactivated possibly via age-related protein(s). Understanding of the detailed molecular signature of intrinsic and extrinsic factors will generate new knowledge on the homeostatic regulation and aging of stem cell maintained organ systems, and open new avenues to control normal HSC function at will and possibly allow specific eradication of pre-malignant stem cells in aged individuals.

Poster Board Number: F-2121

INTRA-AORTIC CLUSTERS UNDERGO ENDOTHELIAL TO HEMATOPOIETIC PHENOTYPIC CHANGES IN EARLY EMBRYOGENESIS

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Intra-aortic clusters (IACs) attach to floors of large arteries and are considered to have just acquired hematopoietic stem cell (HSC)-potential in vertebrate embryos at early mid-gestation. How IACs form and function in HSC generation is largely unknown. To address this issue, IACs were characterized by immunohistochemistry and flow cytometry in mice. Immunohistochemistry analysis showed that IACs were CD31/CD34/c-Kit-positive. The first IACs were identified in the omphalomesenteric artery at 9.0 days post-coitum (dpc). As embryos developed from 9.5 to 10.5 dpc, CD45 expression in IACs increased, whereas that of VE-cadherin decreased, based on flow cytometry analysis, suggesting that IACs lose endothelial phenotypes during this period. To further characterize IACs, we isolated IACs based on CD45 expression. Although no significant differences in hematopoietic potential were observed between CD45-negative and CD45-positive IACs, as demonstrated by colony formation and transplantation, the expression level of hematopoietic transcription factors was highest in CD45-low positive IACs. These results suggest that the transition from endothelial to hematopoietic phenotype of IACs occurs from 9.5 to 10.5 dpc, regardless of hematopoietic potential.

Poster Board Number: F-2122

QUANTITY AND QUALITY CONTROL CULTURE SYSTEM ENFORCES THERAPEUTIC POTENTIAL OF CLINICAL DIABETIC CD34+ CELLS FOR WOUND HEALING

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Background: The quality and quantity of endothelial progenitor cells (EPC), i.e., CD34+ cells is known to be impaired in diabetic patients, thereby raising delayed wound healing at the post transplantation of autologous EPC therapy. In murine diabetic wound healing model, we have recently disclosed the newly developed quantity and quality control culture (QQc) system to recover the vasculogenic property of EPCs for wound healing. Herein, we investigate the effect of QQc on clinical diabetic patient's CD34+ cells, and the therapeutic potency of post QQc-CD34+ cells for diabetic wound healing. Methods: CD34 + cells were isolated from 25 ml of peripheral blood in diabetic patients by auto MACS system, then underwent QQc for 7 days using STEMSPAN serum free medium with VEGF, Flt-3 ligand, TPO, IL-6 and SCF. The vascular regeneration capability of CD34 + cells pre- or post QQc was evaluated with EPC colony forming assay (EPC-CFA). Then, pre- or post- QQc CD34 + cells (2x104/wound) were transplanted into the bed of the stented 6-mm wounds in Balb/c nude mice. Wound healing was assessed by morphometric analysis of the % wound closure, but also histological analysis of granulation, epithelial thickness, and vascular formation by CD31 immunohistochemical staining. Results: EPC-CFA disclosed the predominant generation of functional definitive EPC-CFU in post QQc CD34+ cells vs pre QQc CD34+ cells (definitive EPC-CFU No./1000:3.5±0.6 for post- QQc CD34+ cells vs 1.2 ±0.2 for pre QQc- CD34+ cells, $P < 0.05$, n=4). Transplantation (Tx) of post QQc CD34+ cells consecutively unveiled the greater closure, as compared with that of pre QQc CD34+ cell Tx or PBS (% wound closure: 94.64±2.2 for post- QQc CD34+ cell Tx vs 61.45±5.2 for pre QQc CD34+ cell Tx or 55.67±4.4 for PBS at day 21, $P < 0.05$, n=4). Further, post- QQc CD34+ cells promoted wound granulation and maturation as well as vascularity compared to pre- QQc CD34+ cells or PBS at day 21 (wound granulation pixels/hpf: 386837±84945 for post- QQc CD34+ cell Tx vs for 224295±17688 pre QQc CD34+ cell Tx or 194620±25254 for PBS ; epithelial thickness (μm):18.75±5.2 for post- QQc CD34+ cell Tx vs 11.33±5.6 for pre QQc CD34+ cell Tx or 6.5±3.0 for PBS; micorvascular density (CD31 positive cells/hpf): 34.64±4.2 for post- QQc CD34+ cell Tx vs.43±2.6 for pre QQc CD34+ cell Tx or 20.38±1.7 for PBS1, $P < 0.05$, n=4). These findings indicate that QQc system improves the vasculogenic and regenerative potential of diabetic CD34+ cells for wound healing. Conclusion: The quantity and quality culture system of CD34+ cell provides the methodological clue to overcome the insufficient efficacy of autologous CD34+ cell therapy for wound healing in diabetic patients.

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INDUCED PLURIPOTENT STEM CELL MODEL OF SEVERE CONGENITAL NEUTROPENIA WITH HAX1 GENE DEFICIENCY

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Induced pluripotent stem (iPS) cells are reprogrammed somatic cells with embryonic stem (ES) cell-like characteristics. As iPS cells can be generated from somatic cells of patients with a certain disease, they are expected to be a novel model for disease investigation and drug screening. Recently, we established a neutrophil differentiation system from human iPS cells (Morishima T, et al. J Cell Physiol. 2011). In an attempt to apply the system as a disease model of neutrophil-affected disorders, we generated iPS cells from a severe congenital neutropenia (SCN) patient with HAX1 gene deficiency. The patient was an 11-year-old boy with severe congenital neutropenia onset in 2 months old. DNA sequence analysis revealed a homozygote HAX1 gene mutation in exon 2 (Matsubara K, et al. Haematologica. 2007). Four iPS cell lines were generated from skin fibroblasts of the patient by retroviral transduction of the three or four transcription factors. These patient-derived iPS cell lines showed human ES cell like morphology, expressed typical hu-

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man ES cell markers, and were capable of differentiating into three germ layers by teratoma formation *in vivo*. The transgenes used for the iPS cell generation were confirmed to be well silenced in all iPS cell lines. DNA sequencing analysis of the iPS cell lines identified the same mutation carried in the patient, thus confirmed that we had established the HAX1 deficiency patient-specific iPS cells (HAX1-iPSCs). Next these HAX1-iPSCs and the healthy-person derived iPS cells (WT-iPSCs) were differentiated into neutrophils in vitro using feeder-free culture protocols established in our laboratory (Niwa A, et al. PLoS One. 2011). In this culture system, small human iPS cell clumps were cultured on the matrigel-coated dish with recombinant cytokines and without any feeder cells and fetal calf serum. Around day 25 of culture, myeloid lineage cells including mature neutrophils were obtained as floating cells. Morphologically, the majority of HAX1-iPSCs-derived cells were classified into myeloblast or promyelocyte stage and there were only few mature neutrophils. The proportion of mature neutrophils was only less than 10% in HAX1-iPSCs-derived cells whereas more than 40% in WT-iPSCs-derived cells. Flow cytometric analysis revealed that the percentage of immature CD34 positive cells was significantly higher and that of myeloid-committed CD11b positive cells was significantly lower in the HAX1-iPSCs-derived cells than WT-iPSCs-derived cells. Immunocytochemical analysis for neutrophil specific granules showed that lactoferrin- and gelatinase-positive cells significantly decreased in the HAX1-iPSCs-derived cells compared with WT-iPSCs-derived cells, confirming that HAX1-iPSCs-derived cells contained less mature neutrophils than WT-iPSCs-derived cells. HAX1 is known as an apoptosis-associated protein and neutrophils of the patients with HAX1 gene deficiency are reported to be susceptible to apoptosis. Also in our iPS cell model, annexin V assay revealed that HAX1-iPSCs-derived cells showed higher percentage of apoptosis compared with WT-iPSCs-derived cells. Overall, these HAX1 deficiency patient-specific iPS cell lines recapitulate the hematological phenotype in the patient. These results indicate that patient-derived iPS cells provide us a novel disease models and they can make a contribution to understanding of the pathophysiology and development of novel drugs for the neutrophil-affected disorders.

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FATE TRACING OF E7.5 FLK1+ EXTRAEMBRYONIC MESODERM

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All hematopoietic and endothelial lineages are derived from Flk1+ cells. In embryos, Flk1 is expressed mainly in the extra-embryonic mesoderm at E7.5 stage. Strong Flk1 expression is detected in the allantois and boundary between extra- and intra-embryonic regions including anterior region of intra-embryonic mesoderm (developing cardiac crescent), whereas low expression is seen in the yolk sac blood island region. There is almost no expression in the intra-embryonic region. Lack of etv2, which is indispensable to hematopoietic and endothelial lineages, leads completely disappearance of extra-embryonic Flk1+ mesodermal cells, suggesting that they could be main source of hematopoietic and endothelial lineages. The hematopoietic factors, such as Runx1 and Gata1, are also strongly expressed in the yolk sac blood island at E7.5 stage. Comparing between Runx1 and Gata1 expression, Gata1 expression is more restricted in the yolk sac blood island than Runx1 expression. Runx1 is expressed over the yolk sac blood island. Thus, based on the expression patterns of Flk1, Gata1 and Runx1, E7.5 Flk1+ extra-embryonic mesodermal cells could be classified to at least 3 populations; Runx1+Gata1+Flk1-/low cells, Runx1+Gata1-Flk1+ cells and Runx1-Gata1-Flk1+ cells. To confirm the developmental fate map of these three populations, especially about hematopoietic and endothelial lineages because the differentiation potential at this stage remains poorly understood, we decided to make their fate map using tamoxifen-inducible lineage tracing system. We prepared two mouse lines; Runx1-SACRE and Gata1-MCM mouse lines. Using these two mouse lines, we can trace two Runx1+ populations; Runx1+Gata1+ cell progenies should be traced using Gata1-MCM mice. Comparing the distribution of Runx1-labeled progenies with that of Gata1-labeled progenies enables us to follow the developmental fate map of Runx1+Gata1- cell progenies indirectly. Here we will present our new findings in this attempt.

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HEMATOPOIETIC STEM CELL FATE CONTROL BY GADD45 GAMMA

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Hematopoietic stem cells (HSCs) provide the life-long regeneration of all blood cells. Despite advanced knowledge about the hematopoietic differentiation hierarchy, the molecular network of intrinsic and extrinsic cell fate control needs further elucidation. We identified the stress response gene Growth arrest and DNA damage-inducible 45 gamma (Gadd45γ) as a differentiation and early lineage choice inducer in hematopoiesis. In contrast to reports in other cell types, where Gadd45γ mainly functions as growth suppressor and apoptosis activator, we neither observed these cell fates in Gadd45γ expressing hematopoietic stem and progenitor cells. HSCs lentivirally transduced with Gadd45γ demonstrated an exclusive and accelerated differentiation into the myelomonocytic lineage at the expense of megakaryocyte and erythrocyte cell fate. Single point mutations disrupting Gadd45γ protein homodimerization avert these effects. The other family members Gadd45α and Gadd45β also accelerated HSC differentiation, however, contrary to Gadd45γ, allowing also megakaryocyte and erythrocyte development. The knock-down of Gadd45γ delayed HSC differentiation and remained a pool of immature cells *in vitro*. In blood reconstitution assays, Gadd45γ expressing HSCs, although homing to the bone marrow after transplantation, immediately start their differentiation program and completely lose their self-renewal capacity. Continuous HSC observation by time-lapse imaging and single cell tracking revealed an instant differentiation induction upon Gadd45γ expression within 24h. These HSCs may be even instructed into the myelomonocytic lineage. Cell cycle and survival is not changed in these cells. Further, the hematopoietic cytokines IL-3 and IL-6 strongly induce Gadd45γ expression in multipotent progenitors linking extrinsic signals with the intrinsic lineage choice machinery. Global changes in epigenetic and gene expression patterns by all three isoforms are expected and currently under investigation. Our data suggest that Gadd45γ controls the balance of HSC self-renewal and differentiation, and is able to mediate lineage choice instruction by linking extrinsic and intrinsic determinants.

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MT1-MMP REGULATES HEMATOPOIESIS THROUGH HIF-MDIATED CHEMO-/CYTOKINE RELEASE FROM THE BONE MARROW NICHE

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Stem cells reside in a physical niche, a particular microenvironment. The organization of cellular niches has been shown to play a key role in regulating normal stem cell differentiation, stem cell maintenance and regeneration. Various stem cell niches have been shown to be hypoxic, thereby maintaining the stem cell phenotype, e.g. for hematopoietic stem cells (HSCs) or cancer stem cells. The bone marrow (BM) niche is a rich reservoir for tissue-specific pluripotent HSCs. Proteases, such as matrix metalloproteinases (MMPs) can modulate stem cell fate due to their proteolytic or non-proteolytic functions (abilities). We have investigated the role of membrane-type1 matrix metalloproteinase (MT1-MMP), known for its role in pericellular matrix remodeling and cell migration, in hematopoiesis. MT1-MMP is highly expressed in HSCs and stromal cells. In MT1-MMP-/- mice, release of kit ligand (KitL), stromal cell derived factor-1 (SDF-1/CXCL12), erythropoietin (Epo) and interleukin-7 were impaired resulting in erythroid, myeloid and T and B lymphoid differentiation. Addition of exogenous rec. KitL and rec. SDF-1 restored hematopoiesis *in vivo* and in vitro. Further mechanistic studies revealed that MT1-MMP in a non-proteolytic manner activates the HIF-1 pathway, thereby inducing the transcription of the HIF-responsive genes KitL, SDF-1 and Epo. These results suggested MT1-MMP as a critical regulator of postnatal hematopoiesis, which as a modulator of the HIF pathway alters critical hematopoietic niche factors necessary for terminal differentiation and or migration. Thus, our results indicate that MT1-MMP as a key molecular link between hypoxia and the regulation of vital HSC niche factors.

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MATERNAL SPECIFC IMPRINTING IN THE H19 IGF2 LOCUS MAINTAINS QUIESCENT LONG TERM HEMATOPOIETIC STEM CELLS.

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Precise epigenetic regulation of imprinted genes, via monoallelic differential DNA methylation, is critical for appropriate mammalian embryonic development. However, whether this regulation plays a role in mammalian adult stem cells is unknown. Here we show an unexpected enrichment of maternally expressed growth-restricting imprinted genes in long-term hematopoietic stem cells (LT-HSCs) and their downregulation upon HSC activation and proliferation. Imprinted genes enriched in LT-HSCs included cell cycle arresting genes (*Cdkn1c/p57*or *Kip2, Ndn, H19, Rb1)* and growth restricting genes *(Gtl2 and Grb10)*. In contrast, imprinted genes that were predominantly expressed in ST-HSCs and MPPs were associated with growth promoting functions and activation of stem cells. These included *Ascl2*, *Peg12* and *Sfmbt2* genes. One of the critical locus (*H19-Igf2* locus), known for its role in growth control during embryonic development was differentially expressed between LT and ST HSCs. This was accompanied by changes in methylation in differential methylation region (DMRs) of *H19-Igf2* locus. To explore a potential role of the *H19* Differential Methylation Region (*H19-DMR*) in maintenance of LT-HSC quiescence in adult hematopoietic system, allele specific conditional deletion of *H19-DMR* was performed. Conditional deletion of the *H19-DMR* from the maternal allele (m$H19^{\Delta DMR/+}$), but not from the paternal allele (p$H19^{\Delta DMR/+}$), led to reduction in HSC quiescence and resulted in sequential loss of LT-HSCs and short-term (ST) HSCs with increasing age, and resulted in bone marrow (BM) exhaustion under stressed conditions. Mechanistically, deletion of the m*H19-DMR* resulted in a profound perturbation of the imprinted gene network (IGN) and enhanced Igf2-Igf1r signaling that suppressed FoxO function. Our findings reveal a novel role of maternal versus paternal allele-specific epigenetic regulation of imprinted genes in the long-term maintenance of adult HSCs.

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TURNING SOMATIC CELLS INTO HEMATOPOIETIC STEM OR PROGENITOR CELLS: DEVELOPMENT OF A METHODOLOGY BYPASSING THE NEED OF AN INTERMEDIARY PLURIPOTENT STATE

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The hematopoietic system, because it allows complete ablation of "diseased" cells and full reconstitution after "healthy" hematopoietic stem cell (HSC) transplantation, is of great interest for cell-based therapies. Indeed, HSCs hold great promises for multiple therapies, such as treating a variety of acquired/genetic disorders, and targeted cancer therapy. Currently, human therapies using hematopoietic progenitor/stem cells (HPC/HSCs) are limited due to the fact that the only available HPC/HSCs derived from Bone Marrow (BM) and Umbilical Cord Blood (UCB) present limited expansion potential. Moreover, the availability of human leucocytes antigen (HLA)-matched HSCs continue to hamper their use in large-scale clinical applications. Thus, the generation of an "unlimited number" of human HPC/HSCs or functionally equivalent cells is highly demanding from a clinical perspective. To date, two major strategies have been preferentially studied, both facing limitations: i) expand human HSCs for long term hematopoietic reconstitution; ii) derivation of HSC-like cells from embryonic stem (ES) or induced pluripotent stem (iPS) cells. Indeed, despite intense researches over the past fifty years, the field struggles to develop an efficient and reproducible protocol that allows the generation of functional HSCs suitable for clinical application. Here, we reasoned that ES cell factors can establish active marks at tissue specific elements before being replaced by cell type-specific factors as cells are going under differentiation. In this line, OCT4 has been previously demonstrated to be able to drive fibroblasts towards a hematopoietic-like state. Using an original experimental design we analyzed the feasibility to use embryonic factors, without going through a pluripotent state, in combination with defined culture conditions supporting HSCs differentiation/maintenance/expansion and co-transfection of miRNAs highly expressed in "real" human HSCs. We thus initially developed a method allowing the generation of CD34+/CD45- cells as well as CD34+/CD45+ cells from both fibroblasts and mesenchymal stem cells, without the need of pluripotent cells. Noticeably, the converted cells present changes in their gene expression profile

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showing a switch towards the hematopoietic lineage. Importantly, preliminary *in vivo* data showed the capacity of newly and "early-stage" generated cells (CD34+/CD45-) to engraft into irradiated NOD/SCID mice. Interestingly, Flow cytometry and qPCR analyses have shown that human engrafted cells underwent a maturation step *in vivo* as demonstrated by the acquisition of a CD34+/CD45+ signature 10 weeks post-transplantation. However, those cells failed to entirely reconstitute the hematopoietic system. To further improve the protocol and with the aim to functionally mature the "engraftable" cells we are currently testing the effect of additional miRNAs (e.g. Hsa-miR-10a, Hsa-miR-99a, etc.) in our initial conditions. Additionally, comparative analysis with iPS-derived CD34+ cells, generated from OP9 co-culture system, are under progress and should reveal how different are the cells generated by our new method.

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IN-VIVO TRACKING OF TRANSPLANTED PERIPHERAL MONONUCLEAR CELLS LABELED WITH MANGANESE USING MAGNETIC RESONANCE IMAGING

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Non-invasive high resolution imaging is required to track the cells and evaluate clinical relevance after cell transplantation. Manganese (Mn2+) is known to be an essential element for living organisms and there has been a recent renewed interest in Mn2+ as a useful contrast agent for magnetic resonance imaging (MRI). In-vivo tracking of transplanted peripheral mononuclear-cells (MNCs) with MRI has attempted to visualize the long term regenerative therapeutic effects and the localization of migrated cells at high spatial resolution in living tissues such as skeletal muscle, heart, and brain. We successfully established a method to label MNCs with Mn2+ (Mn-MNCs) and performed longitudinal observation of Mn-MNCs in a rat model of ischemic leg or myocardial infarction with 7-tesla MRI. Mn-MNCs or saline were intramuscularly injected into a border zone of the ischemic lesion and were evaluated by T1-weighted imaging, repeatedly. The transplanted Mn-labeled MNCs were typically detected as a "double-layered" structure having a negative core with surrounding positive enhancement immediately after the intramuscular administration, and those were clearly observed for up to 21 days in T1-weighted MRI. This Mn-enhanced method enables visualization of the transplanted area at 150-175 μm in-plane spatial resolution and allows the migration of labeled-MNCs to be observed for long periods in the same animal. In addition, manganese deposition was not detected when Mn-labeled mononuclear cells were transplanted into brain striatum. Mn2+-enhanced cellular MRI promises to be a useful technique for the quantitative evaluation of cell tracking.

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CROSSTALK BETWEEN THE FANCONI ANEMIA AND NOTCH SIGNALING PATHWAYS REGULATES HEMATOPOIETIC STEM CELL DIFFERENTIATION

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Hematopoietic stem cells (HSCs) can either self-renew or differentiate into various types of cells of the blood lineage. Little is known about the signaling pathways that regulate this choice of self-renewal versus differentiation. Here we report that deregulation of Notch signaling skews HSC differentiation in Fanconi anemia (FA), a genetic disorder associated with bone marrow failure and progression to leukemia and other cancers. In mice expressing a transgenic Notch reporter, deletion of the *Fanca* or *Fancc* gene enhanced Notch signaling in multipotential progenitors (MMPs), which was correlated with decreased quiescent long-term HSCs and increased formation of myeloid progenitors. Furthermore, we found a functional correlation between Notch signaling and self-renewal capacity in FA HSCs. Significantly, we show that FA deficiency in MMPs deregulates a complex network of genes in the Notch and canonical NF-[[Unsupported Character - Symbol Font ]]B pathways, leading to enhanced myeloid differentiation due in part to increased responsiveness to inflammatory environment and transcriptional deregulation of some of the classical Notch target genes including *Hes1* and *Hey1*. Consistent with this, TNF-[[Unsupported Character - Symbol Font ]] stimulation enhanced Notch signaling of FA LSK cells, leading to decreased HSC quiescence and compromised HSC self-renewal. Finally, genetic ablation or pharmacologic inhibition of NF-[[Unsupported Character - Symbol Font ]]B reduced Notch signaling in FA MMPs to nearly wide-type level and partially restored FA HSC quiescence and self-renewal capacity. Taken together, these results demonstrate a functional interaction between the FA pathway and Notch signaling in HSC differentiation and establish a role of FA proteins in regulation of the production of myeloid progenitors, hence preventing myeloid malignancies.

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INDUCTION OF HEMATOPOIETIC CHIMERISM BY ANTI 3RD PARTY CENTRAL MEMORY CD8 T-CELLS AS A PLATFORM FOR IMMUNE TOLERANCE: IMPLICATIONS FOR REGENERATIVE MEDICINE

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Induction of tolerance for organ transplantation through hematopoietic chimerism is a major goal in transplantation biology. Recently the relevance of this critical challenge was extended to regenerative medicine. Despite the success of directing differentiation of EPS or iPS into cell types of therapeutic value, the immune system of the recipient remains a substantial obstacle for their downstream clinical use, even when using iPS generated from the patients' own cells. This unexpected problem, recently demonstrated in animal models, is likely associated with imperfect differentiation, that leads to presentation of embryonic antigens on iPS derived tissues. Given that the success of regenerative medicine is predicated on the resolution of these immunological issues, the development of safe strategies for immune tolerance induction is desirable not only as a prelude for allogeneic organ transplantation,

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but also for transplantation of EPS or iPS derived tissues. Induction of donor-specific tolerance for organ transplantation, which facilitates engraftment in the absence of continuous immunosuppression, can be achieved by establishment of hematopoietic chimerism. One potentially safe and attractive approach is engrafting allogeneic T-cell-depleted BM (TDBM) under reduced intensity conditioning (RIC), thus eliminating the risks of graft-versus-host disease (GVHD) and high toxicity. However, overcoming TDBM rejection by surviving host anti-donor T-cells (HADTC), remains a challenge. In this study we examined the prospect of using donor-derived central-memory CD8+ T-cells (Tcm) directed against 3rd-party antigens, to support TDBMT engraftment under RIC and to induce subsequent tolerance to donor organ grafts, while also exploring their mechanism of action. Our results demonstrate that fully allogeneic or (hostxdonor)F1 Tcm support donor chimerism (>6 months) in sublethally irradiated (5.5Gy) Balb/c mice. Thus, while 14/14 recipients of Nude-B6 BM cells rejected the graft, 9/9 and 15/19 recipients of BM treated with 5X106 allogeneic or F1 Tcm, respectively, exhibited significant donor chimerism. Notably, administration of allogeneic anti 3rd-party Tcm was not associated with any symptoms of GVHD. Reducing irradiation to 4.5Gy was achieved by combining Tcm with short-term low-dose Rapamycin. Importantly, this chimerism resulted in successful donor skin engraftment (>4 months follow-up), while third-party skin was rejected. A novel bioluminescence imaging model for tracking HADTC revealed that homing of Tcm to the LNs induced HADTC accumulation at the LNs, concomitant with their elimination from other organs, further enabling TDBM engraftment. The events leading to this tolerance were further elucidated by 2-photon microcopy showing trafficking arrest of patrolling HADTC in LNs, upon conjugate formation with their cognate Tcm, resulting in apoptosis of HADTC in the LNs. Thus, anti 3rd-party Tcm support TDBMT engraftment under RIC via a unique apoptotic mechanism, without the risk of GVHD. This approach results in donor-specific tolerance, and offers a novel and potentially safe treatment for non-hematologic disorders and a platform for organ transplantation. Furthermore, upon optimization of current approaches for the differentiation of EPS or iPS into pluripotent hematopoietic stem cells, our newly developed protocol for chimerism induction could pave the way for the wide use of EPS or iPS derived tissues in regenerative medicine.

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EX VIVO GENERATION OF GRANULOCYTES UTILIZING A NOVEL RETINOID AGONIST

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Neutropenia is the most feared complications of cancer chemotherapy. It is characterized as a hematological disorder with abnormal low number of neutrophils and results in severe risk of life-threatening sepsis in neutropenic patients. Granulocyte transfusion, using *ex vivo*-generated granulocytes from normal human primitive hematopoietic CD34+ cells, has been considered as a potential therapeutic strategy to reduce the neutropenic threats posed by bacterial and fungal infections. However, the efficiency of *ex vivo* generation of granulocytes is low while the true therapeutic potential of such cells still remain unknown. All-trans retinoic acid (RA) mediates granulocytic differentiation of CD34+ cells through inducing retinoic acid receptor alpha (RARα)-dependent transcriptional regulation of granulopoiesis, and RA treatment of acute promyelocytic leukemia (APL) represents the most successful granulocytic differentiation therapy in modern clinical oncology. We recently discovered that Am80 (tamibarotene), a synthetic novel retinoid agonist designed to avoid side effects of RA, was much more potent than recombinant granulocyte-colony stimulating factor (G-CSF) but less toxicity than RA in generation of *ex vivo* granulocytes (EVG) from CD34+ cells. Moreover, similar to normal human granulocytes, the peripheral blood neutrophils (PBN), Am80-induced EVG displayed much higher bactericidal ability than those induced by G-CSF. This effect was associated with Am80-induced co-expression of CD66 and β2 integrin, CD11b, on EVG. Our studies therefore suggest that Am80 is a cost-effective therapeutic candidate for *ex vivo* generation of EVG by mediating RARα-dependent transcriptional regulation of granulopoiesis, likely through a novel regulation of CD11b that exerts differential effects upon CD66-dependent activity of EVG.

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BMI1 CONFERS RESISTANCE TO OXIDATIVE STRESS ON HEMATOPOIETIC STEM CELLS

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The polycomb-group (PcG) proteins function as general regulators of stem cells. We previously reported that retrovirus-mediated overexpression of Bmi1, a gene encoding a core component of polycomb repressive complex (PRC) 1, maintained self-renewing hematopoietic stem cells (HSCs) during long-term culture. However, the effects of overexpression of Bmi1 on HSCs in vivo remained to be precisely addressed. In this study, we generated a mouse line where Bmi1 can be conditionally overexpressed under the control of the endogenous Rosa26 promoter in a hematopoietic cell-specific fashion (Tie2-Cre;R26StopFLBmi1). Although overexpression of Bmi1 did not significantly affect steady state hematopoiesis, it promoted expansion of functional HSCs during *ex vivo* culture and efficiently protected HSCs against loss of self-renewal capacity during serial transplantation. To understand the mechanism through which Bmi1 maintains HSC function, we first examined the role of Bmi1 in the DNA damage response. Unexpectedly, overexpression of Bmi1 had no effect on DNA damage response triggered by ionizing radiation. We next hypothesized that Bmi1 might play an important role in the regulation of reactive oxygen species (ROS). ROS are endogenously produced through cellular metabolism and function as signaling molecules that regulate a variety of physiological processes. Dysregulation of ROS signaling homeostasis results in oxidative stress, leading to various pathological effects including dysfunction and depletion of HSCs. Recent studies have demonstrated that HSCs are more susceptible to oxidative stress compared to differentiated hematopoietic cells. Of note, Tie2-Cre;R26StopFLBmi1 HSCs tolerated oxidative stress better than the control and maintained a multipotent state, although overexpression of Bmi1 had no impact on the level of intracellular ROS. ROS reportedly activate p38 MAPK stress signaling pathway and upregulate the expression levels of p16 and p19, which are directly repressed by Bmi1. This eventually results in the depletion of HSCs. Bmi1 has the consensus phosphorylation site of p38 within the C-terminal PEST domain, which is highly conserved among vertebrate Based on this evidence, we hypothesized that an increased level of ROS in HSCs somehow attenuates Bmi1 function through the p38 MAPK stress signaling pathway, leading to HSCs exhaustion.. In vitro kinase assay showed that p38 but not JNK could directly phosphorylate Bmi1. Next

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we generated a threonine to alanine mutation at the amino acid position 275 (T275A) within the consensus site of murine Bmi1, to test whether this threonine residue is a major phosphorylation target for p38. We found that the p38-mediated phosphorylation of Bmi1 T275A was significantly decreased compared with wild type. HSCs transduced with Bmi1 T275A showed enhanced cell growth. We are now analyzing the effect of this mutation in HSCs using competitive reconstitution assays. Our findings demonstrate that overexpression of Bmi1 confers resistance to stresses, particularly oxidative stress, onto HSCs, thereby enhancing their regenerative capacity. This suggests that Bmi1 is located downstream of a ROS signaling pathway and negatively regulated by it, possibly through the p38 signaling pathway.

Poster Board Number: F-2136

MYELOID LINEAGE COMMITMENT IN HEMATOPOIETIC STEM CELLS

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Hematopoietic stem cells (HSCs) have self-renewal potential and the capacity to differentiate into all types of blood cells. The Ly5 antigen, which is only expressed by leukocytes, is commonly used to distinguish between donor, recipient and competitor cells. However it is not expressed by platelets and erythrocytes hence the contribution of these populations are not accounted for when assessing multi-lineage reconstitution. Lineage commitment has long been considered to occur at the differentiation stage downstream of multipotent progenitors (MPP). Here we hypothesized that it can occur earlier at the level of HSCs. To assess lineage commitment by HSCs more precisely, we have generated a transgenic mouse line expressing the fluorescent protein Kusabira Orange (KuO) (B6-Ly5.1) in all types of blood cells including platelets and erythrocytes. Highly enriched HSCs, $CD150^+CD41^-CD34^{-/low}c\text{-}Kit^+Sca\text{-}1^+Lin^-$ ($CD150^+CD41^-CD34^-KSL$) cells and $CD150^-CD41^-CD34^-KSL$ cells were individually isolated from KuO transgenic mice. These were transplanted into lethally irradiated mice (B6-Ly5.2) along with 2×10^5 competitor bone marrow cells (B6-Ly5.1/Ly5.2). Secondary transplantations were carried out by transplanting 5×10^6 bone marrow cells from the primary recipient mice into another lethally irradiated recipient. The peripheral blood cells in both primary and secondary recipient mice were periodically analyzed. Long-term full-lineage reconstitution was deemed to have been achieved by the single transplanted cells if full-lineage reconstitution was observed 20 weeks after secondary transplantations. When full-lineage reconstitution was not observed at this time point, the cells were deemed to have achieved short-term full-lineage reconstitution. In mice transplanted with single $CD150^+CD41^-CD34^-KSL$ cells, there were five different types of reconstitutions that were detected. These included the long-term full-lineage type, short-term full-lineage type, the common myeloid progenitor (CMP)-type reconstitution, the megakaryocyte erythroid progenitor (MEP)-type reconstitution, and the megakaryocyte progenitor (MkP)-type. On the other hand, when a single $CD150^-CD41^-CD34^-KSL$ cell was transplanted instead, short-term full-lineage reconstitution and CMP-type reconstitution were detected. These results suggested that these myeloid committed progenitors (CMPs, MEPs, and MkPs) were close to HSCs in the developmental pathway. To determine whether HSCs directly give rise to these progenitors, we next performed paired daughter cell assays. After a single $CD150^+CD41^-CD34^-KSL$ cells divided once in culture, daughter cells were separated by micromanipulation techniques and individually transplanted into lethally irradiated mice along with 2×10^5 competitor cells. Interestingly, we detected HSC-HSC, HSC-MkP, and HSC-CMP pairs in the paired daughter cells, suggesting that MkP and CMP commitment may occur at the level of HSCs through a single division of HSCs. In conclusion, our results demonstrated that the $CD150^+CD41^-CD34^-KSL$ population contains MkPs and CMPs, which HSCs can give rise to via one division. Here, we propose a revision of the hierarchical overview of normal hematopoiesis, namely the direct differentiation from HSCs into myeloid lineage committed progenitors (MkPs and CMPs).

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PHOSPHODIESTERASE-2A REGULATES HAEMATOPOIETIC STEM CELL (HSC) TURNOVER AND ERYTHROPOIESIS

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Chemical mutagenesis of mice combined with advances in hematopoietic stem cell reagents and genome resources can efficiently recover recessive mutations and identify genes essential for generation and proliferation of definitive hematopoietic stem cells and/or their progeny. We used FACS to quantify rare haematopoietic stem cell and progenitor cell subsets in the fetal liver of ENU-mutagenized mice bred to homozygosity. We used a SNP based whole genome scan coupled with exon capture and next generation sequencing of the genetic interval of interest to rapidly identify the causative mutation in two mouse strains (called Kamu and Mulkirri), that have a similar recessive phenotype characterized by a five fold increase in stem cells and anemia. The strains harbour different mis-sense mutations in the pde2a gene, a dual cAMP and cGMP phosphatase. The mutations in exon 23 and exon 26 lead to mis-sense mutations in residues known to be involved in dimerization and function of the phosphatase domain, which demonstrate the power of ENU to find functionally important residues in proteins. The stem cell phenotype will be presented in detail. Pde2a is a novel regulator of stem cell turnover which could be targeted by specific drugs to enhance stem cell function or turnover in vitro or *in vivo*.

Poster Board Number: F-2138

DISTINCT HEMATOPOIETIC FUNCTIONS OF HEMATOPOIETIC CELL SUBSETS IN THE ADULT MAMMALIAN BONE MARROW

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Osteolineage cells (OLCs) modulate hematopoietic stem cell function within the bone marrow microenvironment. We tested

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whether these cells contribute to hematopoietic homeostasis by an acute post-natal *in vivo* cell depletion strategy with ~50% efficiency. Depletion of OLCs expressing Osterix, led to decreased B cell production due to an IL7- and IGF1-dependent differentiation blockade. In contrast, deletion of cells expressing Osteocalcin was associated with decreased T cell precursors, reduced Delta-like 4 ligands, and decreased Notch signaling. Strikingly, in neither short-term setting was hematopoietic stem cell (HSC) number or function compromised. The effects on B and T lymphopoiesis were entirely microenvironment dependent. This study indicates that HSC are insensitive to short-term changes in OLC number while B and T cell lymphopoietic programs are highly dependent upon the integrity of OLC. Selective steps of B and T lymphopoiesis are highly sensitive to specific and distinct OLC and the regulatory proteins they produce.

Poster Board Number: F-2139

M-CSF INSTRUCTS MYELOID COMMITMENT OF SINGLE HEMATOPOIETIC STEM CELLS

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The ability of cytokines to instruct lineage fate in HSC has been highly debated. The question is difficult to address by analysing the behaviour of a bulk population. A definite answer requires the analysis of single rigorously defined HSC before the completion of the first cell division. Towards this end we have characterized the cell fate changes of single highly purified stem cells in response to cytokine signalling during this critical period in culture and *in vivo*. We have shown previously that M-CSF can simulate myeloid commitment of early stem and progenitor population depending on their sensitivity to M-CSF receptor signalling (Cell. 2009, 138(2):300-13). We have now analysed the change of gene expression profile in single individual cells before the first division of M-CSF stimulated highly purified (CD150+CD34-,CD48-,KSLF) HSC. The cytokine induced multiple changes in gene expression consistent with an instructive function of M-CSF on early myeloid commitment of highly purified HSC in vitro. We further demonstrated this was also true *in vivo*. To monitor myeloid commitment we have analyzed activation of PU.1 expression. This transcription factor is an ideal marker of early cell fate change as it is both required and sufficient to drive myeloid fate in early multipotent stem and progenitor cells. We found that highly purified PU.1 negative HSC could change fate and activate PU.1 within 24h *in vivo*, in a normal hematopoietic environment. To demonstrate the commitment of highly purified HSC to PU.1+ cells was dependent on M-CSF signalling *in vivo*, we used blocking antibody or chemical inhibitor to specifically block M-CSF receptor signalling in HSC. Our results demonstrate that cytokine signalling can indeed instructs cell fate changes in individual highly purified long term hematopoietic HSC. Interestingly deficiency for the myeloid transcription factor MafB specifically increased M-CSF instructed cell fate change and PU.1 activation in highly purified (CD150+CD34-,CD48-,KSLF) HSC stem cell compartment capable of HSC with long term high efficient reconstitution capacity but not in any other immature multi-potent progenitor or short term HSC compartment, both in vitro and *in vivo*. Together this indicates that cytokines can instruct lineage fate in HSC. Our data also suggest that a self enforcing circuit of a transcription factor such as PU.1 and a cytokine such as M-CSF that is under the control of a highly specific threshold setter such as MafB can integrate cell autonomous and extrinsic signals in lineage commitment.

Poster Board Number: F-2140

THE LANDSCAPE OF THE HEMATOPOIETIC SYSTEM: AN IN SILICO APPROACH

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Hematopoiesis, the formation of blood cells from hematopoietic stem cells (HSC), is a well-studied differentiation system, including more than 50 different cell types. Recent advances in high-throughput technologies and computational analysis allow us to apply genome-wide modeling to hematopoiesis. The aim of our study is to investigate the relationships between the various hematopoietic lineages, including HSC and committed progenitor cells, and their terminally differentiated progeny. By using a large collection of microarray data, including in-house and public microarray data, we aim at (i) reconstructing the "landscape" of the hematopoietic system and (ii) modeling the gene networks, which drive hematopoiesis. In previous work we used non-negative matrix factorization (NMF) to assess pluripotency of human embryonic stem cells (ES cells) and induced pluripotent stem cells (iPS cells; Mueller et al., Nature 2008; Mueller et al. Nature Methods, 2011). We have now applied NMF on a compendium of human microarray data to model the landscape of the hematopoietic system. Antigen presenting dendritic cells (DC) are so far not well positioned in the conventional fate maps of hematopoiesis and this calls for a revised hematopoietic lineage map (Zenke and Hieronymus, Trends Immunology, 2006). NMF places DC and their specific subsets in a distinct position in the vicinity of B lymphoid and myeloid cells, and distant from T cells, NK cells and granulocytes. In addition, DC appear to arise from HSC via their own developmental pathway, which is different from or only partially overlaps with the pathways of the other hematopoietic lineages. These data open the perspective of (i) elucidating specific gene networks that drive DC development and (ii) testing the impact of such gene networks in gene-knockout models.

Mesenchymal Stem Cell Differentiation

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HIGH-THROUGHPUT ANALYSIS IMPLICATES NOVEL MECHANOSENSITIVE FOCAL ADHESION PROTEINS IN REGULATING HMSC DIFFERENTIATION

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Force-sensitive, cryptic binding site-containing proteins are a common element within the focal adhesion complex and are known to transduce signals necessary and sufficient to induce human mesenchymal stem cell (hMSC) differentiation on substrates of varying stiffness, e.g. the talin-vinculin complex activation is force-dependent and regulates myogenesis on matrices mimicking muscle stiffness. A Cysteine Shotgun/Western Blot (CS/WB) method was used to examine the extent of differential unfolding and exposure of cryptic kinase sites across focal adhesion proteins in response to stiffness to identify novel stem cell mechano-sensors, e.g. talin showed increasing cryptic site vinculin binding as substrate stiffness increases, confirming *in situ* that talin unfolds in response to increasing cytoskeletal force. Using the Scansite Motif Scanner,

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which predicts binding site accessibility, 465 cryptic kinase sites on 47 proteins in the adhesome were found. A detailed analysis of vinculin, which was identified with CS/WB, found that it contains a MAP Kinase 1 (MAPK1) binding site in its cryptic hinge region, a feature common to 21 other focal adhesion proteins. While force-dependent MAPK1 binding to vinculin specifically regulates myogenesis, monotonically increasing talin unfolding with stiffness would suggest broader force-sensitive cell regulation; given this disparate behavior, we developed a 96 well plate hydrogel assay with varying stiffness to assess how a targeted siRNA screen of these other 21 proteins affects hMSC spreading, migration, proliferation, and myogenesis. The data from both the bioinformatics analysis and the siRNA screen lend support to the heretofore unexplored role of MAPK1 in substrate-stiffness based hMSC differentiation, as well as the role of kinases in mechanically induced myogenesis.

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SUBSTRATE STIFFNESS DEPENDENT VINCULIN ACTIVATION MODULATES MECHANOSENSITIVE STEM CELL DIFFERENTIATION

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Human mesenchymal stem cells (hMSCs) in extracellular matrices (ECM) with stiffnesses mirroring that of specific native tissues will differentiate spontaneously towards that tissue type, e.g. muscle stiffness induces myogenesis. While cell fate is regulated by cytoskeletal contractility and transmitted to the ECM via focal adhesions, a consensus on the necessary and sufficient signaling pathways for mechanosensing that converts biophysical forces into chemical signals to induce differentiation has yet to be reached. Here we assess whether focal adhesion proteins may differentially initiate signaling cascades by acting as 'molecular strain gauges,' undergoing force-dependent conformational changes that create new binding sites. One such strain gauge, talin, unfolds under physiological force to expose vinculin binding sites, suggesting that vinculin may play a role in mechanosensitive signaling. Small interfering RNA (siRNA) was used to achieve a 75% reduction in total and focal adhesion-localized vinculin levels, confirmed by western blot, qPCR, and immunofluorescence. As a result of this knockdown, we observed an 80% decrease in stiffness-induced MyoD, a muscle transcription factor. Moreover, knockdown impaired hMSCs' ability to sense mechanical differences between soft and stiff ECM, thus blocking stiffness-sensitive migration or "durotaxis." To determine whether vinculin knockdown perturbed focal adhesion structure, altered adhesive properties, or diminished cellular force generation, immunofluorescence on other focal adhesion proteins, a spinning disc adhesion assay, and 3-D traction force microscopy were performed, respectively, with little data indicating global changes in focal adhesions. To identify mechano-sensitive vinculin domains sufficient for stiffness-induced differentiation, vinculin was selectively deleted and specific domains subsequently added back into knocked-down cells. Rescue of MyoD expression was observed for specific domains relating to kinase signaling required for myogenesis. Together, these data suggest the first *in situ* evidence that force-sensitive focal adhesion proteins can activate stem cell differentiation signals.

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TRANSPLANTATION OF HEPATIC CELL SHEETS FROM HUMAN BONE MARROW-DERIVED MESENCHYMAL STEM CELLS WITH A SMALL MOLECULE COMPOUND AMELIORATES ACUTE LIVER FAILURE IN MICE.

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Backgounds & Aims: Bone-marrow derived mesenchymal stem cells (BM-MSCs) have not only a capacity for hepatic differentiation but also have regenerative effects by producing trophic factors. Therefore, cell-based therapies utilizing BM-MSCs are expected to be an alternative treatment for liver transplantation. We have showed that Wnt/β-catenin signaling was inhibited during hepatic differentiation of BM-MSCs, and that BM-MSCs, of which Wnt/β-catenin signal is downregulated by siRNA or small molecule compounds, differentiated toward hepatic lineage. For development of the therapeutic tool for liver disease, hepatic cell sheets from human BM-MSCs with Wnt/β-catenin signal-suppressing small molecules were transplanted into mice with acute liver failure. Methods: Cell sheets were fabricated by temperature-responsive polymer (PIPAAm)-grafted culture dishes substituted for conventional culture dishes. UE7T-13 BM-MSCs were plated onto φ60mm PIPAAm-grafted culture dishes at the cell density of 9.0×10^3 cells/cm^2, and on the following day the culture with 0.8 μM hexachlorophene, a Wnt/β-catenin signal-inhibitor, was started up to 8 days, and stopped by reducing the dish temperature to 20°C for 20 min. NOD/SCID mice were divided into four groups; group 1, 2 and 3 in which monolayer, 2 layers and 3 layers cell sheets were transplanted onto two sites of liver surface, respectively, and in group 4 sham operation was done. All groups were orally administrated 2.0 μl/g body weight of carbon tetrachloride one day after transplantation. Serum aminotransferase and bilirubin were measured on 2, 4 and 8 days after transplantation. Finally, all the mice were sacrificed on day 8 and examined by RT-PCR analysis and immunohistochemistry. Survival curve was also examined by a log-rank test. Results: Although serum aminotransferases were gradually decreased on 2, 4 and 8 days in all groups, serum AST were significantly decreased in group 3, 2, 1 in this order, compared to that on day 4 ($p<0.01$, each), and serum ALT was decreased almost in the same way ($p<0.01$, each). Significant reduction of bilirubin on day 2 was observed in group 3, 2 and 1 in this order, compared to group 4 ($p<0.01$, each). Furthermore, survival rate was remarkably improved in group 2 and 3, compared to group 4 ($p<0.05$). RT-PCR analysis showed that several human-specific humoral factors such as IL6, bFGF, HGF, α1-antitrypsin, apolipoprotein E and complement 3 were expressed in the grafts of group 1, 2 and 3. Conclusions: Transplantation of cell sheets manipulated by this approach into acute liver failure mice resulted in the reduction of hepatic injury and improvement of survival rate. Hepatic cell sheets have dual effects, one of which inhibits liver damage and the other of which compensates liver-specific functions. This technology has the great potential as a promising therapy for acute liver failure.

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IDENTIFICATION OF SMALL MOLECULES INDUCING HEPATIC DIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS THROUGH INHIBITION OF WNT/B-CATENIN SIGNALING

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Backgrounds and Aims Mesenchymal stem cells (MSCs) are expected to be a potential cell source for regenerative medicine. We have already showed that Wnt/β-catenin signaling was downregulated during hepatic differentiation of human bone marrow-derived mesenchymal stem cells (BM-MSCs) and human umbilical cord blood-derived mesenchymal stem cells (UCB MSCs). In addition, downregulation of Wnt/β-catenin signaling in MSCs drives their differentiation toward hepatic lineage. Since small molecules are more safely and stably applicable for clinical settings than nucleic acids or protein drug products, we attemted to identify the potent compounds accelerating hepatic differentiation of MSCs. In the present study, we focused on the derivatives of known Wnt/β-catenin signal inhibitors. Methods First, we synthesized twenty three compounds, derivatives known as inhibitors of Wnt/β-catenin signaling pathway; one derivative from hexachlorophene, two derivatives from PKF118 310, three derivatives from ICG-001, three derivatives from NSC668036 and fourteen derivatives from PNU-74654. Second, the availability of these derivatives was examined in UE7T-13 BM-MSCs and UCBTERT-21 UCB-MSCs. The effects of these compounds on cell viabilities and TCF4/β-catenin transcriptional activities were assessed by WST assay and luciferase reporter system, respectively. The compounds with downregulating TCF4/β-catenin signal were further examined for hepatic differentiation as follows; the culture of UE7T-13 and UCBTERT-21 cells plated at a cell density of 9.0×10^3 and 2.0×10^3 cells/cm^2 was started, and on the following day, culture media were replaced with fresh media containing each compound. The same cell density was adjusted every 8 days, and the culture was maintained for 24 days. Hepatic cells-derived from MSCs were examined on hepatocyte-specific markers by RT-PCR and immunofluorescence, and on hepatic functions by PAS staining and urea assay every eight days. Results Six compounds suppressed Wnt/β-catenin signaling pathway, and five of six molecules induced albumin mRNA in UE7T-13 cells. Among them, IC-2, a derivative from ICG-001, was the most potent inducer of hepatic differentiation of UE7T-13 cells, judging from RT-PCR analysis, immunofluorescence and urea assay. On the other hand, hepatic differentiation of UCBTERT-21 cells was not so strongly, but surely induced by two derivatives from PNU-74654. Conclusion We identified novel small molecules which efficiently induce hepatic differentiation of human BM-MSCs and UCB-MSCs. However, these molecules were more effective in differentiating ability in BM-MSCs than in UCB-MSCs. These molecules are expected to be useful for clinical application in regenerative medicine for liver disease.

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THE HUMAN AMNIOTIC FLUID STEM CELLS HAVE THE POTENCY TO DIFFERENTIATE INTO GERM CELLS

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Approximately 1% of women under the age of 40 years and 0.1% under the age of 30 years experience premature ovarian failure (POF). POF is an ovarian defect characterized by the premature depletion of ovarian follicles with unexpected menopausal symptoms and infertility. Human amniotic fluid is known to contain multiple cell types derived from the developing fetus and can give rise to diverse differentiated cells without ethical and religious concerns associated with human ES cells. So far, the lack of data describes the derivation of germ cells from human amniotic fluid cells (hAFCs). In this study, we characterize the germ cell expression in hAFCs samples. As expected as previous report, hAFCs consistently express human OCT4 and Nanog gene, but less expresses the other germ cell marker. During the hAFCs culturing, we observed clones formation and identify that over 67% of the clone cells expressed the transcription factor OCT4 and the surface antigen c-Kit (CD117) by flow cytometry, whereas only 28% of the attached differentiated hAFCs expressed Oct-4 and c-Kit ($P<0.01$). This clone cells could be induced to embryonic bodies (EBs) in differentiation condition medium and the markers, including OCT4, Nango decreased with differentiation, whereas only Blimp1 and Stella increased by real-time PCR and immunostaining. Compared with this, the germ cells marker Blimp1, Stella, DAZL, VAZA, Stra8, Scp3, Scp1, and GDF9 highly expressed in EBs in differentiation conditions with 5% human follicular fluid (FF) and germ cell maturation factor cocktail (FAC) for 1 week, whereas OCT4, Nanog decreased with differentiation. These results showed that clone cells from hAFCs have the potential to differentiate to germ line in vitro. To test the functional potential of hAFCs to develop to oocytes *in vivo*, we transplanted clone cells derived from hAFCs infected with GFP lentil-virus into ovaries of infertile mice by pre-treatment with cyclophosphamide and busulphan to destroy the existing pre- and post-meiotic germ cell pools. Two months after hAFCs transplantation, ovaries were collected and the presence of oocytes was determined by their morphological appearance and expression of GFP. Histological evaluation showed that ovaries injected with hAFCs had many oocytes at all stages of development, including GFP-positive oocytes. However, immature oocytes or follicles were not found in control ovaries form recipients without hAFCs transplantation Furthermore, the control ovaries consisted of stromal, interstitial cells and a few atretic follicles. Anti-Müllerian hormone also known as AMH has a role in folliculogenesis and is a useful marker of ovarian function. Our results showed that AMH expression is strong in granulosa cells of preantral and small antral follicles, and gradually diminishes in the subsequent stages of follicle development in normal ovaries. AMH is no longer expressed in ovaries sterilized by chemotherapy. However, AMH expression appears in recipient ovaries after hAFCs transplantation for 2 months. These results suggest that oocytes can be regenerated and ovarian function can be partly restored in sterile recipient female mice by transplantation of hAFCs. Taken together, we highlight new possibility for use of human amniotic fluid stem cells in regenerative medicine in the arena of reproductive health.

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FIRST DEMONSTRATION THAT HUMAN VERY SMALL EMBRYONIC-LIKE (VSEL) STEM CELLS CAN REGENERATE HUMAN BONE IN A MODEL OF SKELETAL REPAIR

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Human VSELs (hVSELs) are a resident population of pluripotent stem cells in the bone marrow (BM) involved in the normal turnover and regeneration of tissues. The levels of VSELs in peripheral blood are greatly increased in response to injury, and they have been shown to repair injured tissues. hVSELs are SSEA-4+/CD133+/CXCR4+/Lin-/CD45-, express pluripotency markers (Oct-4 and Nanog) and may be able to differentiate into cells from all three germ lineages. Our aim was to develop a cell-based autologous therapy for skeletal tissues using hVSEL cells. hVSELs isolated from peripheral blood by apheresis following G-CSF mobilization were fractionated and enriched by elutriation and FACS. Collagen sponge scaffolds containing 200-30,000 hVSELs cells were implanted into cranial defects generated in SCID mice. Analysis by μCT showed that a population containing VSELs produces mineralized tissue in the cranial defect compared to controls at 3 months. Histological studies show significant bone formation and cellular organization within the cranial defects compared to cellular or scaffold controls alone. The extent of osteogenesis appeared to be inversely dependent on the number of cells seeded, with the cell populations containing 2,000 VSEL cells seedings demonstrating more intact cortex-like structure, denser thickness of trabeculae and more bone marrow than those of 10,000 cells, consistent with complex interactions that may be occurring between and amongst stem cells and accessory cells. Staining with antibodies to human leukocyte antigens proved that the newly generated tissues were of human origin. To further understand the *in vivo* differentiation capabilities of hVSEL cells, histologic sections from purified and enriched hVSEL implants were stained with antibodies specific for human osteocalcin (a specific mature osteoblastic marker), human nestin (a neural marker), and human PPARγ (adipocyte). Human specific PPARγ shows positive immunostaining of cells in sections of purified and enriched VSELs groups. Staining with human specific nestin antibodies showed positive staining along large cellular tracks that were highly suggestive of neuronal formation. In addition, it was observed that blood vessel endothelial cells stained with human specific HLA co-localized with hCD31. We also examined whether these VSELs migrated away from the defect site by quantitative real-time PCR for human specific Alu sequences. The results demonstrated that there were low levels of human cells in the blood, but that the cells did not migrate from the site of implantation into other murine tissues. This study demonstrates for the first time that human VSEL cells can potentially generate human bone in a mouse model of skeletal repair. These studies lay the foundation for future cell-based regenerative therapies for osseous and connective tissue disorders including trauma and degenerative conditions such as osteoporosis, fracture repair and neoplastic repair.

Poster Board Number: F-2147

WNT TENASCIN-C REGULATES NEUROTROPHIN-INDUCED NEURONAL TRANSDIFFERENTIATION OF HUMAN BONE MARROW-DERIVED MESENCHYMAL STEM CELLS IN 3D PELLET CULTURES

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Adult human mesenchymal stem cells are generally restricted in their differentiation potential to the tissue where they reside. However, we previously reported that tenascin-cytotactin (Tn-C)/Wnt-7a upregulates brain-derived neurotrophic factor (BDNF)/neurogrowth factor (NGF)/retinoic acid (RA) triggered-transdifferentiation of human mesenchymal stem cells (hMSCs) toward neuronal progenitor-like cells in 2D culture. Adding TnC/Wnt to DMEM incorporated with BDNF/NGF/RA enables hMSCs to increase neuron marker expression, including microtubule-associated protein-2 (MAP2), glial fibrillary acidic protein (GFAP), βIII tubulin, and neurofilament (NFM). In the 3D pellet culture, Tn-C upregulates the mRNA of MAP2, and NFM is compared to the 2D culture, but downregulates the glial cell marker GFAP. Adding Tn-C with Wnt 7a enables hMSCs to increase MAP2, NFM significantly; in contrast to Tn-C only, cells increase GFAP expression in 3D cultivation. We further found that Tn-C enhances neuronal lineage of integrins α5 and α7, but inhibits osteo-chondrogenic lineage, integrin α2. In contrast, Tn-C combined with Wnt 7a inhibits the neuronal lineage of integrins α5 and α7, and enhances osteo-chondrogenic lineage, integrin α2. In conclusion, Wnt 7a acts as a proliferative stimulator, but Tn-C plays an important role in the neuronal differentiation of hMSCs in 2D and 3D cultivation. These findings are vital in stem-cell therapy for nerve regeneration. Keywords: mesenchymal stem cell, Wnt-7a, neurogenesis, pellet, tenascin C, transdifferentiation

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THE ROLE THY-1 IN HUMAN MESENCHYMAL STEM CELLS

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Mesenchymal stem cells (MSC)s are the adult tissue multipotential stem cells that give rise to cells of the skeletal connective tissues (osteoblasts, chondrocytes, adipocytes and hematopoiesis-supportive stromal cells). A couple of observations suggest that they can also give rise to skeletal muscle cells, cardiac muscle cells and endothelial cells. In addition, some reports indicate that they may give rise to non-mesodermic derivative cells such as hepatocytes (endoderm) and neurons or astrocytes (neuro-ectoderm).There is increasing interest in the therapeutic potential of human MSCs. The cell-based therapies and studies have focused on the use of MSCs derived from human bone marrow, lipoaspirate, dental pulp and cord blood. Such therapeutic treatments have induced recovery and stimulated endogenous tissue regeneration programms in many organs. However, the main limitation of this approach is the large number of cells needed for transplantation associated with the observation that isolated MSCs can not undergo many passages *ex vivo* without loosing their morphological and multipo-

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tential- differentiation properties. The efficiency in the differentiation rate of MScs into some lineages is still a challenge in the field. The imunophenotypic characterization of MSCs shows that they are positive the presence of the cell markers Thy-1 (CD90), CD105, CD73, CD117, CD44, CD166, CD29 and STRO-1; and negative for the markers CD45, CD34, CD31 e CD14. Although, the markers Thy-1 and STRO-1 are not specific for MSCs, these are the two cell marks broadly used to identify MSCs. Thy-1, or CD90, is a glycoprotein present in the cell membrane of thymocytes, T cells, natural killer cells, neurons, numerous adult stem cells (MSCs, hematopietic stem cells, pancreatic stem cells, hepatic stem cells and muscle progenitors cells), glomelular mesengial cells, ovarian cancer cells, nasopharyngeal carcinoma cells, endothelial cells and fibroblasts. Although it has been shown to be conserved among different species, the function of Thy-1 seems to differ depending of the cell type. Its role in adult stem cells is still unknown, however, it has been suggested that it is involved in cell adhesion, cell proliferation and migration. Our group has previously observed that the expression of Thy-1 decrease as the cell commits to the differentiation pathways, suggesting that Thy-1 can be used as a marker for the undifferentiated status of MSCs. Here, we report the effects of the knockdown of the expression of Thy-1 in MSCs derived from human dental pulp and cord blood. Lentiviral particles were used in order to create MSCs lineages that stably produce siRNA against Thy-1 and a scrambled siRNA control. The ablation of Thy-1 was confirmed in flow cytometry assays and the effects of this knockdown were determined by performing growth curves, flow cytometric imunophenotyping and differentiation assays. We found that a significant decrease in the levels of Thy-1 does not affect the proliferation of MSC. However, the ablation of Thy-1 accelerates and increases the MSCs ability to differentiate into the osteogenic and the adipogenic lineages. Further studies in progress will elucidate whether the ablation of Thy-1 expression also facilitates the differentiation of MSCs into other lineages and whether it affects MSCs therapeutic properties.

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GENERATING BROWN FAT FROM ADULT HUMAN BONE MARROW STEM CELLS

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Today the world is facing an obesity pandemic. Obesity is a major risk factor for diabetes, cardiovascular diseases and some cancers. Current treatments for obesity mainly aim at reducing the energy intake side of the equation and have met limited success. Brown adipose tissue (BAT) has the potential to increase the body's energy expenditure through non-shivering thermogenesis, due to the presence of uncoupling protein 1 (UCP1). When activated by β-adrenergic stimulation, BAT has a large capacity for glucose disposal and triglyceride clearance. Originally believed to only be present in infants, the rediscovery of BAT in adult humans promises much hope for therapeutic intervention to treat obesity and related diseases. However, no readily available source of brown adipocyte progenitors for humans exists yet. Adult human bone marrow mesenchymal stem cells (hbmMSCs) upon adipogenic induction differentiate into white adipocytes by default. However using macromolecular crowding (MMC) as a novel cell culture platform, MMC is able to drive brown adipocyte differentiation of adult hbmMSCs, suggesting that hbmMSCs may be a novel cell source for brown adipocyte progenitors in humans. MMC enhanced the expression of UCP1 by over 20-fold in hbmMSC-derived adipocytes induced with a brown protocol compared with non-MMC controls induced by a classical white protocol. The presence of UCP1 is detected on a protein level as well. Data suggests that the generated brown adipocytes are functional as they respond to forskolin and isoproterenol stimulation by 1) upregulating UCP1 by a few hundred fold and other thermogenic genes such as PGC1α and DIO2, 2) increasing mitochondrial mass and 3) increasing lipolysis. We hope that this technology would serve as a platform for several applications: 1) a screening tool for pharmacological agents that can promote the differentiation of brown adipocytes, 2) a viable adult cell source to study BAT formation in adult humans and 3) a cell-based therapy to generate a brown fat pad for the treatment of obesity and related diseases.

Poster Board Number: F-2150

CHANGES OF HUMAN SKIN-DERIVED MSC PHENOTYPE BY MODELING NEURAL STEM CELL NICHE CONDITIONS *IN VITRO*

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Background. Skin-derived mesenchymal stem cells (S-MSCs) are multipotent stem cells that have the capacity to differentiate into mesodermal and neuroectodermal cell lineages. S-MSCs can be induced to express neuronal and glial lineage specific markers in the presence of appropriate growth and differentiation factors. However, it is still unknown whether the functional role of S-MSCs is to replace neurons or glia or rather to act as the modulator of the neural stem cell niche. The modulating effect could be reached by either inducing changes into the extracellular matrix (ECM) composition and/or changing the cell phenotype. In the present study we were interested to find out whether S-MSCs up-regulate expression of neurogenesis associated ECM and cell adhesion proteins when propagated in the neural progenitor expansion medium. We also investigated the changes of the S-MSCs phenotype by modeling neural stem cell niche conditions *in vitro*. Methods. S-MSCs were seeded on laminin β1 or fibronectin coated plates in the following cell culture media: 1) control medium DMEM supplemented with 10% FBS; 2) neural progenitor expansion medium - DMEM/F12 3:1, 2% B27, FGF and EGF (both 20 ng/ml); 3) neuronal differentiation medium - Neurobasal, 2% B27, 1% N2, NT-3, BDNF (both 10 ng/ml) and 4) glial differentiation medium - DMEM/F12 3:1, 2% B27, 50 ng/ml neuregulin and 4uM forskolin. The medium was changed every 2-3 days and the duration of the experiment was three weeks. First, the expression of ECM, cell adhesion and neuroectodermal markers in S-MSCs was analysed by PCR. We used three primer panels: 1) ECM panel collagen a1, fibronectin 1, Laminin β1, 2) cell adhesion panel integrin β1, integrin β8, integrin α5, integrin α6; 3) neuroectodermal panel nestin, tubulin bIII, MAP2, S100B, ENO2, GALC, Olig1 and Olig2. Next, cells were analyzed weekly for neuronal marker tubulin β III, glial marker GFAP, S100B and SOX-10 expression by immunofluorescence analysis. Additionally, p75NTR expression was analyzed by flow cytometry. Results. Undifferentiated S-MSCs expressed ECM genes fibronectin 1, Laminin β1, collagen a1; cell adhesion molecules integrin β1, integrin β8, integrin α5, integrin α6 and neural genes nestin, tubulin β III, MAP2, ENO2, GALC. Quantitative PCR data revealed more than 3-fold increase of laminin β1, 5-fold increase of collagen a1, 2-fold increase of integrin β1 and nestin after three week propagation in neural progenitor expansion medium. Immunophenotype analysis showed that S-MSCs in

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control medium expressed neural markers nestin, tubulin β III, GFAP and failed to express SOX-10 and S100B. After one week propagation in neuronal differentiation medium the expression of p75NTR was induced. Additionally, we observed strong SOX-10 expression whereas S100B expression was low. Interestingly, the SOX-10 expression was low and S100B was undetectable in the S-MSCs that were cultivated in glial differentiation medium. Conclusion. Our results indicate that S-MSCs increase the expression of laminin and integrins in the neural progenitor medium. More interestingly, S-MSCs expressed markers characteristic to immature Schwann cells p75NTR, S100B, GFAP, SOX10 when propagated in the presence of neurotrophic factors BDNF, NT-3 on fibronectin and laminin coating. Altogether our data suggest that S-MSCs most likely act as modulators of the dermal niche that could support regeneration of the peripheral nerve tissues.

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MIMICKING THE BONE-MARROW NICHE: CONTINUOUS CULTURE OF FRESH BONE MARROW ASPIRATES IN HYPOXIA ENHANCES OSTEOGENIC DIFFERENTIATION OF HUMAN FETAL MESENCHYMAL STEM CELLS

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Mesenchymal stem cells (MSC) are osteogenic and clinically attractive for use in bone tissue engineering applications. As the physiological oxygen tension of MSC residing in the bone marrow niche is at 2-7%, we hypothesised that oxygen tension is a critical parameter for maintenance of the stem cell phenotype. Our eventual goal is to investigate the necessity of hypoxic culture for maintaining stemness of MSC, optimising cellular expansion and facilitating therapeutic fracture repair response *in vivo*. Low passage human fetal MSC (hfMSC) expanded under normoxic (21%) conditions previously were cultured in a monolayer to study the effects on growth behaviour and differentiation capabilities under 2% and 21% oxygen tensions over a period of 14 days. Results were compared against hfMSC isolated from human fetal bone-marrow aspirates grown continuously in 2% and 21% oxygen levels from the initial cultures. Initial studies using normoxia-maintained hfMSC showed 1.3 fold increased proliferation ($p<0.01$) from Day 3 and four-fold increase in colony-forming units-fibroblasts (CFU-F) capacity ($p<0.01$) in hypoxia compared to normoxic cultures. Alkaline phosphatase levels were consistently lower between Day 7-14 (4.7-5.3 fold; $p<0.001$), with 3.1 fold reduction in calcium deposition in hypoxic cultures on Day 14 ($p<0.001$). In line with this observation, CFU-F and proliferative potential was lowered when switched from hypoxic to normoxic-cultures as compared to those kept in hypoxia continuously. Here, short-term hypoxia culture of hfMSC maintained self-renewal as seen by its increased growth kinetics, although osteogenic differentiation was impeded in hypoxic cultures. Paradoxically, hfMSC derived in hypoxia and kept in such conditions demonstrated more robust osteogenic capacity, with 2.9 fold higher calcium content on Day 14 ($p<0.001$), and increased CFU-F capacity (6.5 fold; $p<0.05$). Thus, the implementation of continuous hypoxic cultures resulted in the most potent hfMSC isolates in terms of CFU-F, proliferation capacity and osteogenic potential, and is likely to play an important role for bone repair. This culture strategy is thus a promising approach in retaining stem cell properties that is necessary for therapeutic efficacy in the clinical setting.

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SERPINE2 PROMOTES OSTEOGENIC DIFFERENTIATION OF MESENCHYMAL STEM CELLS FROM HUMAN MANDIBLE VIA PI3K/AKT AND ERK PATHWAY

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Human bone marrow stem cells (hBMSCs) are the useful sources for tissue engineering. In this study, we examined the secretome of hBMSC from mandibular bone (Mn-hBMSCs) during early osteogenesis by geLC-MS/MS. We then examined the expression patterns of selected candidates in mRNA levels. Secretion levels of SERPINE1 and SERPINE2 were significantly up- and down- regulated at 3 days of osteogenic induction, respectively. Among these secreted proteins, SERPINE2 mRNA levels were decreased and then elevated 7 days after osteogenic differentiation induction. This pattern of SERPINE2 expression was also confirmed in secreted protein levels during osteogenic diffentiation. To examine the role of SERPINE2 in osteogenesis, we treated the Mn hBMSCs with human recombinant SERPINE2 protein (rhSERPINE2) with osteogenic medium. rhSERPINE2 significantly enhanced mineralization of Mn-hBMSCs. rhSERPINE2 significantly increased phsopho-Akt and phospho-Erk1/2 level. In addition, rhSERPINE2-induced Akt and Erk1/2 activity and ALP activity were blocked by PI3K and Erk inhibitors, respectively. In conclusion, SERPINE2 is supposed to be involved in osteogenic differentiation via PI3-Akt and Erk pathway.

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LNGFR AND THY-1 BASED PROSPECTIVE ISOLATION OF HUMAN MESENCHYMAL STEM CELLS REVEALS FUNCTIONALLY DISTINCT SUBPOPULATIONS

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INTRODUCTION: Mesenchymal stem cells (MSCs) are self-renewing cells with the ability to differentiate into osteocytes, chondrocytes and adipocytes. MSCs are multipotent and have low immunogenicity, are considered as potential candidates for a variety of clinical applications. However, to date, these cells have been poorly characterized, which raises significant concerns, as human trials using MSCs are currently underway. Traditionally, the isolation of MSCs from unfractionated whole bone marrow (WBM) relies on their adherence to plastic dishes. Prolonged culture is often required to remove contaminant cells and obtain a reasonably pure population of MSCs. However, during this culture process, the differentiation potential and proliferative ability of the CFU-Fs gradually diminishes as the cells acquire a more mature phenotype. In the recent study, we describe an innovative method that enables the simple and reliable prospective isolation of MSCs based on their expression of both LNGFR and Thy-1. RESULTS: Multicolor cell sorting and

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CFU-F assays showed that the LNGFR+Thy-1+ cells demonstrated a CFU-F frequency that was about 200,000 times higher than unfractionated WBM cells. Single cell sorting assay of LNGFR+Thy-1+ cells revealed three distinct sub-populations within we have designated rapidly proliferating clone (RPC), moderately proliferating clone (MPC) and slowly proliferating clone (SPC). RPCs demonstrated robust multi-lineage differentiation and self-renewal potency. We observed a greater increase in SA beta-gal positive cells in MPCs and SPCs compared to RPCs, demonstrating that MPC and SPC cells undergo cellular senescence. To examine genomic stability, array-based comparative genomic hybridization (aCGH) was performed to investigate aneuploidy and genomic abnormalities. We found that MPCs and SPCs accumulated non-overlap copy number variations (CNVs) as an indicator of *de novo* genomic DNA abnormalities, although no errors were identified in any of the RPCs studied. Furthermore, a significant increase in p16 expression was observed in MPCs and SPCs compared to RPCs, in contrast to p21 and p14 expression. Therefore, cellular senescence in MPCs and SPCs might be triggered by *de novo* genomic DNA abnormalities followed by p16 expression. Given the characteristics of RPCs, which make them attractive for therapeutic applications, the observation of moderate CD106 and CD49d expression on RPCs after six weeks in culture, compared to the absence of their expression in MPCs and SPCs, was particularly noteworthy. Based on the observation that within cultured LNGFR+Thy-1+ cells the expression of CD106 gradually decreased with time, we then compared the growth of CD106+ and CD106- cells from MSCs and found that CD106+ cells had a far greater CFU-F capacity. Finally, considering that migration is an important functional feature of MSCs, we decided to test the migration ability of the three different populations. A migration assay (with function-blocking antibodies) revealed that RPCs exhibited significantly higher migration ability than MPCs or SPCs. CONCLUSIONS: Clonogenic characterization of LNGFR+Thy-1+ MSCs has demonstrated unexpected cellular heterogeneity, however we show that CD106 can used to select the most potent and genetically stable stem cells (RPCs). These specific cell surface markers may prove beneficial in a clinical setting as they can be used to isolate purified MSCs from fresh bone marrow, as well as after in vitro expansion.

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A BRIEF STUDY FOR THE USE OF HUMAN ADIPOSE DERIVED STEM CELLS AND HOFFA´S FAT PAD IN THE CARTILAGE REGENERATION UNDER DIFFERENT CONDITIONS

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Introduction: Arthritis is a joint disorder featuring inflammation, frequently accompanied by joint pain. The causes of arthritis include injury (leading to osteoarthritis), hereditary factors, infections, etc. Chondrocytes are the cellular component of cartilage but this tissue has a poor intrinsic repair capability due, in part, to its avascular nature. Actually, there are many publications about the capacity of Mesenchymal Stem Cells to differentiate *in vitro* into chondrocytes. In this study we analyzed two new sources of cells, lipoaspirates and Hoffa´s fat pad, and different conditions to determine the best way to get chondrocytes for cell therapy. Material and Methods: Stem cells obtained from human adipose tissue (hASC) and Hoffa´s fat pad (HFP) were cultured under different conditions to test the ability to differentiate into chondrocytes. The medium used to induce chondrogenesis was DMEM high glucose supplemented with 10 ng/mL TGF-β3, 1 mg/mL BSA, 1 X ITS, 5 µg/mL Linoleic Acid, 1 µM Dexamethasone and 5 µg/mL Ascorbic Acid. Cells from both different sources were cultured for 21 days in: i) Normal culture dishes in normoxia and hypoxia; ii) Dishes coated with Collagen I in normoxia and hypoxia; iii) and pellet cultures in normoxia and hypoxia. To determine the chondrocyte phenotype RT-PCR for SOX-5, SOX-6, SOX-9, Collagen I, Collagen II, Collagen IX, Collagen X and Aggrecan I and immunocytochemistry assays for SOX-9 and Collagen II were carried out at day 6, 14 and 21. Results: Collagen I was expressed at the same levels in all cultures even in control however Aggrecan I showed a decrease in its expression in hASC cultured but not in cells obtained from HFP which showed similar expression levels of this marker throughout the process. SOX-5 and Collagen X showed an increase in their expression after 21 days for all cultures but the higher expression levels were detected when cells were cultured in dishes coated with Collagen I in normoxia both for hASC and HFP. Collagen II was not expressed in pellet cultures but low levels were detected in normoxia and hypoxia in the other cultures getting better results in dishes coated with Collagen I under normoxia conditions. Low levels of SOX-9 were detected in all treatments being a little higher after 21 days in both cells linage. Collagen IX was only detected when both kind of cells were cultured in dishes coated with Collagen I under normoxia conditions. Expression of SOX-6 was not detected in any culture using hASC however cells obtained from HFP showed low levels of this chondrogenic marker in all cultures. Immunocytochemistry assays were used to corroborate the expression of SOX-9 and Collagen II determining a higher expression of both markers in cultures maintained for 21 days in dishes coated with Collagen I although no significant differences were detected when were compared cultures in normoxia and hypoxia from both cell sources. Conclusions: Stem cells obtained from lipoaspirates and HFP have the capacity to express different chondrogenesis markers when are cultured with differentiation medium. The best conditions to induce chondrocyte differntiation are the use of dishes coated with Collagen I in normoxia conditions for 21 days. Both kinds of cells have the capacity to differentiate into chondrocytes but only stem cells from HFP have the ability to express all markers studied therefore this cells are considered the best source for chondrocyte regeneration.

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HEPATIC DIFFERENTIATION OF FRESHLY ISOLATED HUMAN BONE MARROW-DERIVED MESENCHYMAL STEM CELLS AND THERAPEUTIC APPLICATION FOR ACUTE LIVER INJURY

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[Aims] Human bone marrow-derived mesenchymal stem cells (BM-MSCs) can differentiate into hepatocytes, and are expected to be an excellent cell source for regenerative medicine. We have identified several small molecules which accelerate the differentiation of human immortalized BM-MSCs into functional hepatocytes by downregulating Wnt/β-catenin signal. As a therapeutic application of BM-MSCs into clinical settings, we first have to differentiate freshly isolated BM-MSCs from human subjects, and second have to demonstrate these cells can be applicable as clinically available modalities. In the present study, we demonstrated that freshly

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isolated BM-MSCs from healthy individuals and the patients with osteoarthritis could be differentiated into hepatocytes by small molecules, and that the combination of small molecules and temperature-sensitive cell sheets could reduce acute liver injury in mice. [Methods] Human bone marrow-derived cells of healthy persons and the patients with osteoarthritis were obtained from Lonza Ltd. and our hospital patietns under informed consent, respectively. Clonally expanded BM-MSCs were sorted by CD90 and CD271. First, the small molecules including hexachlorophene, its derivative HC-2, IC-2, a derivative of ICG-001, and PN3-13, a derivative of PNU-74654 were examined on cell viability. Second, the reporter assay to evaluate the TCF4/β-catenin transcriptional activity was performed using the compound concentrations of 70~90% viability. Third, BM-MSCs were incubated in the presence of each compound, and then expression of albumin, AFP, CK18, GS, CYP1A1, TDO2, TAT, ASGR1, HNF3β and EpCAM were weekly analyzed with RT-PCR. Fourth, the functional assays such as PAS stain and Urea assay were performed. Fifth, the cell sheets made by incubation on the temperature-sensitive dish with IC-2 was transplanted on liver surface of NOD/SCID mice, then carbon tetrachloride was orally administered, and serum transaminases and bilirubin were measured up to 7 days. [Results] Hexachlorophene, IC-2, HC-2 and PN3-13 suppressed TCF4/β-catenin transcriptional activity by approximately 30~50% up to 8 days culture. IC-2, HC-2 and PN3-13 were an inducer of hepatic differentiation of BM-MSCs by RT-PCR. Immunofluorescence analysis demonstrated that these cells were positive for albumin and C/EBPα . Hepatocyte-like cells derived from BM-MSCs which were treated with 40 μM of IC-2 showed glycogen production and urea production. The transplanted cell sheet made by incubation on the temperature-sensitive sheet with IC-2 reduced liver injury. [Conclusion] Our results indicated that downregulation of Wnt/β-catenin signal with small molecules efficiently induced hepatic differentiation of freshly isolated MSCs, and the cell sheet made by incubation with these compounds was effective for acute liver injury in mice. These data suggest that cell sheet technology combined with these molecules is a promising tool for regenerative medicine in hepatology field.

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STEM CELL CHARACTERISTICS OF HUMAN MSCS IN DIFFERENT PASSAGES

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hMSCs (human mesenchymal stem cells/ human marrow stromal cells) are heterogeneous subsets of stromal stem cells that could be easily obtained from human bone marrow. The hMSCs are ready to proliferate into large amount in culture, and they have been studied not only in the laboratory-based studies, but also in clinical trials. However, the hMSCs may undergo senescence during long time *in vitro* culture. Here, we compared stem cell properties and anti-inflammatory properties of hMSCs on different passages to demonstrate the relationship between senescence and function of hMSCs. The different passages of hMSCs (P3, P7 and P10) were prepared by repeat culture-harvesting and were kept in liquid-nitrogen until use. After recovery of hMSCs, the cells were examined colony forming unit (Crystal Violet staining after 14 days culture in 20% CCM) and multiple differentiation ability into osteoblasts (Alizarin Red S staining), adipocytes (Oil Red O staining) and chondrocytes (Toluidine Blue staining) after 21 days culture in individual differentiation inducing medium to demonstrate the stem cell properties. Moreover, the cells were compared epitope of cell surface antigens (CD105, CD90, CD29, CD34, CD271 and CD117) expression by Flow cytometry. To compare anti-inflammatory property, the hMSCs were mixed-cultured with a mouse microglial cell line BV-2 and then exposed to 10 ng/mL interferon γ (IFNγ) for inflammatory activation of BV-2. After 48 hours incubation, the nitric oxide metabolite (NOx) production in media was assessed by Griess assay. P3 hMSCs were found to differentiate clearly into adipocytes, osteoblasts, and chondrocytes determined by histological staining after 21 days culture in individual differentiation medium. To compare with P3 hMSCs, P7 hMSCs showed a decrease of adipocytes differentiation but increased osteoblasts differentiation. The chondrocytes differentiation ability and colony number did not change obviously. Both P3 hMSCs and P7 hMSCs formed about 20 colonies from 100 single cells after 14 days culture. However, P10 hMSCs decreased all the osteoblasts, adipocytes and chondrocytes differentiation ability and colony forming property. The cell surface antigens expression was not significantly changed in each passage of hMSCs. Therefore, it is suggested that the individual aspect of stem cell characteristics of hMSCs lose with passage number but show different pattern of variation. As observed in the mix-culture study, BV-2 cells control group (without hMSCs) showed 77.1 μmol/L NOx production 48 hours after stimulation with INFγ. P3 hMSCs mix-cultured with BV-2 reduced the NOx production by 53.9% (35.6 μmol/L). These may suggest that P3 hMSCs have an anti-inflammation activity. However, either P7 hMSCs or P10 hMSCs showed lower anti-inflammation activity than P3 hMSCs when mix-cultured with BV-2 cells which reduced the NOx production by 32.6% (52.0 μmol/L) and 41.5% (45.1 μmol/L) respectively. Although it is reported that the stem cell properties of hMSCs are decreased by senescence, we demonstrated the decrease of anti-inflammatory property in hMSCs along with senescence. Moreover, we found that adipocytes differentiation ability and immunosuppressive property of hMSCs showed a similar pattern of decline accompanied with senescence.

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HUMAN CRANIAL BONE DERIVED STEM CELLS HAVE THE REMARKABLE CAPACITY TO DIFFERENTIATE INTO NEURAL LINEAGES

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Recently, regenerative medicine has gained significant attention for the treatment of central nervous system diseases. In the last decade, a variety of cell types, including human neural stem cells, embryonic stem cell derivatives, and adult bone marrow stromal cells have been investigated the therapeutic benefits for particular neurological disorders. Bone marrow stromal cells (BMSCs) have suggested having the capacity to differentiate into neural lineages and to be clinically attractive because autologous transplantation can be performed in humans. Although most studies with BMSCs have used those originated from iliac bone, a few papers have dealt with the BMSCs originated from different tissues like tooth-derived stem cells or adipose-derived stem cells, and demonstrated their unique cellular characteristics for the neuro-regenerative activities. Human cranial bones are thought to originate from the cranial neu-

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ral crest that plays important role for constructing central nervous systems in the embryo. No previous paper is reported specifically on the isolation of human cranial bone derived stem cells (HCSCs) and these cellular activities for neural differentiation. Here, we have investigated HCSCs capacities of neural differentiation comparing with human iliac BMSCs (iBMSCs). Bone marrow samples were obtained from the volunteer's fronto-temporal cranial bone wastes in neurosurgical procedure after informed consent according to the hospital's guidelines. The bone marrow samples harvested culture dishes and the medium was exchanged to eliminate floating bone powder and non-adherent cells. Cells were exchanged fresh medium in twice a week and incubated until 90% confluence. The cells adhered to the bottom of the culture dish were used as HCSCs. Flow cytometry analysis showed that HCSCs expressed a set of mesenchymal stem cell markers. Subsequently, the cells were induced to differentiate into neural cells with neurotropic factors. RT-PCR, Western blot, and immnocytochemical staining demonstrated that neural differentiation from HCSCs expressed marker for neural cells remarkably stronger than that from iBMSCs. This is the first demonstration of *in vitro* successfully isolation of HCSCs. HCSCs has stronger potency to differentiate into neural cells than iBMSCs, and would be a novel alternative source of autologous adult stem cells to treat neurological disorders.

Poster Board Number: F-2158

HUMAN EMBRYONIC AND FETAL MESENCHYMAL PROGENITORS DIFFERENTIATE EFFICIENTLY TO SKELETAL MUSCLE AND ENGRAFT *IN VIVO*

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The human body has over 600 skeletal muscles (SkM), collectively accounting for approximately 40% of body weight. This important tissue undergoes a progressive decline in mass and function with aging. While quiescent SkM progenitor cells-the satellite cell-exist in the adult organism, its numbers and regenerative capacity does not appear to be sufficient to compensate for muscle loss due to injury and/or aging. Mesenchymal stem cells (MSC) are adult stem cells that have the potential to differentiate into multiple mesodermal lineages. While adult bone marrow (BM) is the best-studied source of MSCs and has been shown to be capable of SkM differentiation, aging also decreases MSC numbers and can affect differentiation capacity adversely. Moreover, invasive procedures are required to obtain these MSCs, further limiting applicability. We have isolated and characterized several fetal- and developmentally early-stage MSCs from the placenta, bone, and embryonic stem cells (ESCs). These human developmentally early-stage mesenchymal progenitor cells (hDE-MPCs) are easily accessible, and have been shown to possess similar differentiation capacity as BM MSCs while harboring higher proliferative indices. Thus, we hypothesized that hDE-MPCs can efficiently differentiate into the SkM lineage and engraft *in vivo*, using a mouse model of SkM injury. Using MSCs from the term placenta, fetal bone, and human ESCs, we found that all three hDE-MPCs have the potential to undergo SkM differentiation. Under standard muscle-inducing conditions, we found hDE-MPCs differentiating towards a SkM lineage, rather than cardiomyocytic or smooth muscle lineage, as evidenced by increased expression of SkM-associated markers including MyoD and α-actinin at the gene and protein level, respectively, and in vitro formation of myotubes. Moreover, *in vivo* experiment demonstrated that only SkM-differentiated hDE-MPCs can be incorporated into host SkM efficiently after injury. In contrast, adult BM MSCs did not express SkM-associated genes under similar in vitro conditions, nor showed *in vivo* engraftment. We then further investigated which factors would account for these differences in SkM differentiation potential between hDE-MPCs and BM MSCs. We found that hDE-MPCs expressed higher levels of serum response factor (SRF) than BM MSCs, which is a transcription factor responsible for SkM lineage commitment and has been shown to decline with aging. Knockdown of SRF in hDE-MPCs resulted in decreased MyoD and myosin expression, its downstream gene. We speculate, therefore, that the discrepant capacity of various MSCs to differentiate in vitro and engraft *in vivo* into SkM may be due to SRF expression levels related to the different developmental ages of the progenitors. Our results demonstrate that hDE-MPCs can be good candidates for SkM regeneration, and further opens up the use of fetal- and developmentally early-stage tissue as possible sources for cell therapy.

Poster Board Number: F-2159

EVALUATION OF TRANSCRIPTION FACTORS AND CHROMATIN REMODELING COMPLEX RESPONSIBLE FOR CONTRACTION IN CARDIOMYOCYTES DERIVED FROM HUMAN BONE MARROW MESENCHYMAL STEM CELLS

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Human Mesenchymal stem cells (hMSC) are multipotent stem cells and can be isolated from different sources like dental pulp, umbilical cord (UC), bone marrow (BM), adipose tissue etc. Amongst adult stem cells, BM- MSC is considered as one of the best candidates for regeneration of cells because of their properties like easy isolation, robust expansion, immunological naivety and absence of ethical concern. These can be differentiated into osteoblasts, adipocytes, chondrocytes, cardiomyocytes etc. The present study aimed at cardiomyogenic differentiation of hBM-MSC and its evaluation in terms of expression of transcription factors (TF) and chromatin remodeling complex, minimally required for beating. This study was initiated after IEC (Institute Ethics Committee) & Stem cell Ethics Committee clearance and samples were collected after proper informed consent from patients. MSC were isolated from BM on the basis of plastic adherence property and 3rd passage cells were used for differentiation after their characterization by surface markers (CD105, CD29, CD90, CD73 and HLA I & II, CD34/45) studies using Flow cytometry. hBM-MSC were seeded on to 35-mm culture dish at the density of 0.05x106 million cells/ml and were maintained at 37ºC/ 5% CO2. At 50-60% confluency cells were induced with 6 uM of 5-AZA for 24 hours and later expanded in LG-DMEM supplemented with 10% FBS following regular media change for next 30-days. Post differentiation cells were characterized for cardiac specific markers Myosin Light Chain-2v, Myosin Light Chain-2a and cardiac Troponin I by RT-PCR and Immunofluorescence (n=5). Differentiated hMSC were able to demonstrate all major characteristics of cardiac cells except contraction. Therefore, we evaluated the comparative level of TFs, GATA4, Tbx5, Nkx2-5 and chromatin binding complex (Baf60c) in hBM-MSC derived cardiomyocytes (n=5) & compared it with human cardiac biopsy (n=2) taken as positive control (Tetralogy of Fallot). These TFs were evaluated by RT-PCR and further by quantitative PCR (qPCR). (qPCR) data revealed that the TFs are expressed at significantly lower levels when compared to adult beating cardiomyocytes. Of the four TFs studied, the expression of NKX 2-5 was observed to be the lowest in human BM

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MSC differentiated cardiomyocytes by 210 folds. Similarly, GATA4 and TBX5 were also expressed at a lower level but to a lesser extent i.e. by 13.4 and 7.5 folds respectively. BAF60C, a cardiac specific BAF complex subunit was also observed to be expressed at a lower level by 6.35 folds as compared to the beating adult cardiomyocytes. The decreased expression of BAF60C may affect the binding frequency of GATA4 to the promoter of cardiac actin and myosin light chain. Along with GATA4, decreased expression of NKX 2-5 may affect the expression of Myosin heavy chain and Troponin apart from others. This may finally lead to suboptimal level of expression of these genes and subsequent proteins. This might in-turn further effect the complete formation of the protein complex required for contraction. TBX5 which was again expressed at lower level, is considered to be responsible for the expression of HCN, which is a potassium channel playing a major role in the contraction of cardiomyocytes. Thus, this study brings forward one of the very important aspect in the differentiation of adult MSC i.e molecular aspect of contraction and indicates a need for improvisation of the current protocols for cardiac differentiation of BM-MSC.

Poster Board Number: F-2160

TRANSCRIPTION FACTOR NETWORK OF EARLY DIFFERENTIATING HUMAN MESENCHYMAL STROMAL CELLS

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Aging of the human skeleton is characterized by a decrease in bone mass due to increased bone resorption by osteoclasts and uncoupled bone formation by osteoblasts. These changes are more pronounced in postmenopausal women that are more susceptible to develop osteoporosis. A striking phenomenon is the increase of bone marrow adipocytes upon aging and in patients with osteoporosis. As osteoblasts and adipocytes share a common precursor in the bone marrow (Mesenchymal Stromal Cells, MSCs), we wanted to understand the early regulatory events that occur upon differentiation of human MSCs. Identification of early transcription factors and the regulation of target genes could further expand our knowledge of lineage decision and commitment of hMSCs and whether the increased number of bone marrow adipocytes can be reduced by interfering with early lineage specific regulators. To address this question, we have differentiated hMSCs into osteoblasts and adipocytes, isolated RNA and analyzed the transcriptome in a high temporal resolution using Illumina microarrays. Our analyses illustrate that gene expression changes were very dynamic upon differentiation of hMSCs into adipocytes or osteoblast. Furthermore, we show that lineage specific changes occur as fast as 30 minutes after start of differentiation, and reach a stable gene expression state within 2-3 days of osteogenic differentiation that lasts until the onset of mineralization 2 weeks after. Analyses of functional categories showed that gene ontology terms such as tanscription regulation, transcription factor activity and DNA-binding were significantly enriched 3 hrs after initiation of differentiation. In addition, 70% of all TFs that were differentially regulated between osteoblasts and adipocytes in the first 4 days were already significantly regulated within the first day. Using TransFac (Biobase), we found over 100 different transcription factor binding sites (TFBS) that were enriched in the genes that were differential expressed of both differentiating osteoblasts and adipocytes. The combination of TFBS analyses and differentially expressed TFs enabled us to generate a transcription factor network of early osteogenic and adipogenic differentiating human mesenchymal stromal cells. Taken together, we have identified transcription factors that were activated/repressed upon differentiation of bone marrow derived mesenchymal stromal cells and can be key regulators for lineage decision. More in depth analyses to identify early regulatory mechanisms are ongoing and will increase our understanding of lineage decision/commitment of hMSC.

Poster Board Number: F-2161

ISOLATION AND CHARACTERIZATION OF ADIPOGENIC PROGENITOR SUBPOPULATION FROM MOUSE BONE MARROW

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Bone marrow adipogenic progenitors (BMAP) have long been considered as a potential cell source for adipose tissue engineering; recently, they have also been reported to actively participate in bone marrow haematopoiesis regulation and the development of osteoporosis. Thus, there is an increasing need to isolate a pure BMAP population for both clinical and basic scientific research. However, currently there is no reported efficient method or specific surface marker for BMAP isolation. In this study, we successfully applied a novel protocol that involves silca microsphere incubation during bone marrow mesenchymal stem cell (BMSC) purification to isolate a pure BMAP subpopulation from mouse bone marrow. The selected BMAP cells showed homogenous fibroblast-like morphology and exhibited particularly high adipogenic ability, low osteogenic ability and nearly no chrodrogenic ability in vitro. During adipocyte differentiation, most cells (>95%) turned into preadipocyte-like morphology containing obvious lipid droplets within 2 days and rapidly accumulated large lipid droplets within 1 week. After 4-day adipogenic induction, differentiated cells highly expressed adipogenesis related genes (PPAR-y, AP-2, CEB/P, Adiponectin, Glut-4) and can be strongly stained by Oil Red O and adipocyte specific marker-Perilipin. When compared with unselected BMSC after 1-week adipogenic differentiation, BMAP cells exhibited much higher adipogenesis genes expression level and Oil Red O stain extract absorbance, indicating that they possess significantly stronger adipogenic ability than the unselected population. Flow cytometry analysis revealed that both selected and unselected BMSC populations were lin- and CD34- after purification. The unselected BMSC population mainly expressed Sca-1+CD90+CD105+CD73- phenotype, while the selected BMAP subpopulation mainly expressed Sca-1+CD90-CD105+CD73- phenotype. Notably, after long-term in vitro culture (after P12), BMAP cells would gradually lose their high adipogenic ability and their surface markers expression also progressively switched from Sca-1+CD90-CD105+CD73- to Sca-1-CD90-CD105-CD73+. We found that the reduced adipogenic ability (Oil red O extract absorbance) was tightly correlated with the decreased CD105 expression (R=0.92) and to a less extent with the Sca-1 expression (R=0.76). Moreover, sorting of CD105+ cells from the low adipogenic BMAP cells in later passage (P14) retrieved the high adipogenic cells with similar morphology and marker expression as early passage BMAP cells. These data strongly support the expression of CD90-CD105+CD73- should be the marker combination to identify BMAP subpopulation. In conclusion, our study demonstrates BMAP subpopulation can be effectively isolated by a BMSC purification protocol involving silica microsphere incubation. These selected cells exhibit much higher adipogenic ability than the unsorted BMSC and express a specific panel of surface markers. Importantly, sorting of CD105+ cells from later passage cells may restore their high adipogenic ability. More experiments will be

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performed to evaluate the *in vivo* adipose tissue formation ability of this subpopulation and test whether this technique can be applied for adipogenic progenitor isolation from human tissues.

Poster Board Number: F-2162

HUMAN AMNIOTIC FLUID STEM CELL ISOLATION AND DIFFERENTIATION INTO OOCYTE-LIKE CELLS

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Stem cells isolated from human amniotic fluid show the extensive self-renewal ability and grow in highly viable long-term cultures, and these cells are able to differentiate spontaneously into derivatives of all three embryonic germ layers. Previous reports have proved that embryonic stem cells and adult pancreatic stem cells can differentiate into cells resembling germ cells and oocytes. Here, we showed that human amniotic fluid stem (hAFS) cells that were isolated from human amniotic fluid at the 2nd trimester of gestation had the ability to differentiate into oocyte-like cells. The specific cell surface antigens of hAFS cell were characterized by immunocytochemistry, RT-PCR and flow cytometry. One hAFS cell line that has been long-time cultured *in vitro* up to 50 passages was used for the differentiation. In order to initiate the differentiation toward oocyte-like cells, hAFS cells (at 10th passage) were induced in the conditional medium supplemented with porcine ovary extracts. During the induction of hAFS cells, gene expression pattern of several ES markers such as Oct4, MBP-15, stem cell factor (CD117) and Stella were detected. For over 10-day induction, the expressions of Oct4, BMP15, CD117 and Stella were significantly increased. After two-three weeks induction, oocyte markers such as ZP1, ZP2 and ZP3, and the meiosis marker, synaptonemal complex protein 3 (SCP3), could be detected by both RT-PCR and immunocytochemistry assay. We also observed the morphology changes of hAFS cells from the fibroblastoid shape to oocyte-like shape after 14 days differentiation. Those oocyte-like cells surrounding with zona pellucida-like membrane were detached from the cell surface and floated on the medium surface when continue to culture the induced cells for 21 days. RT-PCR assay showed that these oocyte-like cells expressed the FSH receptor (FSHR) and the post-meiotic stage marker VASA which were not identified in uninduced cells. In summary, the morphology change and biological determination indicate that under our conditional culture condition the hAFS cell can differentiate into oocyte-like cells. The biological function of induced oocyte-like cells is under the investigation. Keywords: human, amniotic fluid stem cell, oocyte, conditional medium, differentiation

Poster Board Number: F-2163

MOUSE MSCS INDUCE A CD4CD25FOXP3 TREG SUBPOPULATION DURING THE DIFFERENTIATION PROCESS OF TH1 AND TH17 CELLS

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Background: Mesenchymal stem cells (MSCs) are an interesting nonhematopoietic stem cells population with potential applications in regenerative medicine, and more recently for their ability to regulate immune responses. It has been demonstrated that MSCs are able to suppress T lymphocytes activation through several mechanisms including their ability to generated FoxP3+ regulatory T-cells in vitro and *in vivo*. However, the effect of MSCs on T cell and on mature proinflammatory Th1 and Th17 cells still remains unclear. Objective: The aim of this study was to determine whether MSCs were able to generate CD4+CD25+Foxp3+ regulatory T-cells during the differentiation process and in mature Th1 and Th17 cells. Methods: Mesenchymal stem cells were obtained from mice bone marrow and characterized according to their surface antigens expression and by their multilineage differentiation potential. CD4+ T cells were obtained from mice spleen and induced to differentiate into Th1 or Th17 cells. At D0, D2 and D4 of the differentiation process T cells were activated with anti-CD3/CD28 beads and cultured with MSCs. After 6 days of coculture, the expression of CD25 and the transcription factor Foxp3 were measured by flow cytometry. For the functional assays, the "Tregs" subpopulation generated in the presence of MSCs were cocultured with CD4+ T cells labeled with CFSE in the presence of concanavalin A. After 48 hours, proliferation was measured by flow cytometry. Results: We demostrated that MSCs were able to generated CD4+CD25+Foxp3+ T-regulatory cells during the differentiation of Th1 and Th17 cells (Th1:1,25% to 25% and Th17 2% to a 20%; $p<0,05$)s while no effect was observed on mature Th1 and Th17 cells. We also demonstrated that this population had functional effect and were able to suppress the activation of T-cells (45% to a 21%, $p<0,05$). Conclusion: All these results showed that MSCs are a potent immunosuppressive tool that can be propose for the treatment of autoimmune diseases. However, still more data are need to determined which type of diseases are able to be controlled by MSCs and the precise moment when they must be applied, thus it remains as area of intense investigation.

Poster Board Number: F-2164

TRANSPLANTATION OF ADIPOSE TISSUE DERIVED STEM CELLS AND PLATELET RICH PLASMA FOR OSTEOARTHRITIS: AN ASSESSMENT FOR SAFETY AND EFFICACY IN MOUSE MODEL

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Adipose tissue derived stem cells (ADSCs) combined with platelet rich plasma (PRP) commonly used in preclinical and clinical osteoarthritis treatment. However, this therapy has not been carefully evaluated the safety and efficacy. This research aims to assess the safety and efficacy of ADSCs combined with PRP transplantation. Ten samples of ADSCs and PRPs from donors were used in vitro and *in vivo*. About safety, we evaluate the expression of some genes related to tumor formation such as Oct-4, Nanog, SSEA3, SSEA4, alkaline phosphatase by RT-PCR, flow cytometry and cytochemistry stain of ADSCs and tumor formation when injected in NOD/SCID mice. About efficacy, ADSCs were checked the chondrocyte differentiating potential in vitro when cultured in specific medium and *in vivo* when injected with PRP into murine joint that caused joint failure. The results showed that ADSCs are negative with Oct-4, Nanog, SSEA3, SSEA4, alkaline phosphatase as well as can not cause tumors in mice. ADSCs can differentiate into chondrocytes in vitro as well as *in vivo*. ADSCs combined with PRP can improved the joint regeneration in mice. These results proved that ADSCs combined with PRP transplantation is safe and effective therapy for osteoarthritis treatment.

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Poster Board Number: F-2165

ELECTRICAL STIMULATION ENHANCED NEUROGENIN 2 EXPRESSION OF MOUSE BONE MARROW STROMAL CELLS AND INTENSIFIED THE EFFECT OF CELL TRANSPLANTATION INTO BRAIN INJURY

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Bone marrow stromal cells (BMSCs) have gained significant attention for the cell therapy of central nervous system disease. Even now, demonstrated BMSCs transplantation results were poor in neural defect area. Recently, using gene transfection method mediated more capability of transplantation cells. However, this method occurred canceration or immune rejection. Then, it required induce cell differentiation from BMSCs to the neural cells more effectively and safely for cell therapy. On the other hand, a variety of cellular responses to an electrical stimulation have been reported. It was reported cell differentiation of PC12 cells was electrically induced to extend processes. However, there are few studies using BMSCs exposed electrical stimulation study. Electrical stimulation method can reduce gene transfection during neural differentiation. Therefore, we inducted mouse BMSCs (mBMSCs) to neural cells using electrical stimulation and transplanted the cells into traumatic brain injury (TBI) model mice. We investigated the effect of electrical stimulation on their differentiation and evaluated functional recovery and histological changes after transplantation. mBMSCs were harvested from adult mice and seeded in proliferation medium. After the cells reached 70% confluent, they were inducted to differentiate into neural cells and electrically stimulated (Group ND+E). As control group: cells were induced to neural differentiate without stimulation (Group ND). 7 and 14 days after neural induction, cells were analyzed or transplanted in TBI mice. Cells were intravenously transplanted into them 7 days after injury. Mice brains were removed after 3 weeks of transplantation and examined. In Group ND+E, early neural gene and protein (nestin, Pax6) expressed higher than ND at day 7. At day 14, mature neural markers expressed higher in Group ND+E. These results suggested that electrical stimulation could induce BMSCs into neural stem like cells. Furthermore, strong activation of p38 MAPK was examined in Group ND+E. It is well known that p38 MAPK contribute to cell differentiation. Motor function significantly improved in the 7 days induction cells of Group ND+E transplanted mice. Group ND+E cells survived in mice brain after transplantation, and showed high expressions of neural marker. In contrast, there were a few astrocytic marker positive cells. Motor function recovery of 14 days induction cells of Group ND and Group ND+E transplanted mice was poorly. We showed that the electrical stimulated cells have good potential to contribute to recovery from traumatic brain injury. Moreover, Group ND+E cells expressed neurogenin 2 higher than Group ND at day 7. Neurogenin 2 is involved in neural differentiation and inhibits astrocytic differentiation during cell growth. Present study showed the potential of electrical stimulation to differentiate mBMSCs into neural cells. This stimulation method can reduce the risk of canceration by using gene transfection. This study may contribute to using BMSCs instead of ES cells or iPS cells for CNS disease.

Poster Board Number: F-2166

RODENT DENTAL MESENCHYMAL STEM CELL HOMING IN TISSUE REPAIR

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Mesenchymal stem cells (MSCs) from tooth pulp share many properties with MSCs from other tissues including bone marrow. The ease with which they can be isolated makes them an attractive potential source for therapeutic applications. Rodent incisors grow continuously and we have recently shown that in response to tooth damage *in vivo*, mesenchymal cells (transit amplifying cells) from the cervical end of the incisor are stimulated to migrate towards the site of damage and differentiate into specialised cells to facilitate repair. In order to investigate this cell homing we have studied the migration of these cells in vitro. Cells were isolated from incisor pulp and grown in alphaMEM medium. Growth curves and differentiation experiments using pulp cells from the cervical end (MSC area) and from the molar and incisor body regions (non-MSC area) were performed, and their migration properties were compared using scratch wound healing and transwell migration assays. Our results demonstrate that cells isolated from the mesenchyme close to the cervical loop region are distinctly different from those isolated from bone marrow or molar dental pulp. These differences are evident in terms of size, morphology and proliferation capacity. Cells from the incisor cervical area exhibit migratory behaviour in both the scratch and transwell assays. Directed cell migration towards tooth dentine was evident in vitro. Addition of putative migration stimulatory and inhibitory factors in these assays is being used to identify candidate molecules that are released from the damage site.

Poster Board Number: F-2167

BONE MARROW DERIVED MESENCHYMAL STEM CELLS DIFFERENTIATE TO HEPATIC MYOFIBROBLASTS BY TRANSFORMING GROWTH FACTOR BETA 1 VIA SPHINGOSINE KINASE, SPHINGOSINE 1 PHOSPHATE AND S1P RECEPTORS AXIS

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Sphingosine kinase (SphK) is involved in numerous biological processes such as cell growth, proliferation and differentiation. However, whether it participates in differentiation of bone marrow-derived mesenchymal stem cells (BMSCs) to myofibroblasts is presently unknown. Here, in carbon tetrachloride-treated mice, we found SphK1 was expressed in BMSCs in the damaged liver. Furthermore, mRNA expression of SphK1 and transforming growth factor-beta 1 (TGF-beta 1) was significantly increased after liver injury, with a positive correlation between them. Administration of SphK inhibitor, SKI, significantly blocked BMSCs differentiation to myofibroblasts during liver injury, as the proportion of BMSC-derived myofibroblasts decreased markedly compared with that without SKI treatment, and attenuated the extent of liver fibrosis. Using primary mouse BMSCs, we demonstrated that TGF-beta 1 induced BMSCs differentiation to myofibroblasts, which was accompanied with up-regulation of SphK1 and modulation of sphingosine 1-phosphate (S1P) receptor (S1PR) expression.

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Notably, pharmacological or siRNA-mediated inhibition of SphK1 abrogated the prodifferentiating effect of TGF-beta 1. Moreover, by using S1PR subtype-specific antagonists or specific siRNAs, we found the prodifferentiating effect of TGF-beta 1 was mediated by S1PR1 and S1PR3. These data suggest that SphK1 activation by TGF-beta 1 leads to differentiation of BMSCs to myofibroblasts mediated by up-regulation of S1PR1 and S1PR3, providing new compelling information on the mechanisms by which TGF-beta 1 gives rise to fibrosis and opening new perspectives for its pharmacological treatment.

Poster Board Number: F-2168

COMPARISON OF CARTILAGE DIFFERENTIATION OF RAT MARROW DERIVED MSCS CO CULTURED WITH RAT OSTEOBLASTS AND CHONDROCYTES ENCAPSULATED IN ALGINATE GEL

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Introduction: Mesenchymal stem cells (MSCc) have been considered as surgical therapeutic cell source in order to treat full-thickness articular cartilage defects. The expressions of osteogenic as well as collagen X genes, however, in MSC chondrogenic cultures are the major concern in their application. Interestingly, co-culture systems of marrow-derived MSCs with mature articular chondrocytes (ACs) would be a putative way to efficiently promote MSC cartilage differentiation. Furthermore in skeletal development osteoblasts have been known to provide morphogenic signals that promote cellular differentiation into cartilage lineages. The present investigation is an attempt to evaluate chondrogenic effects of mature osteoblasts (Oss) as well as articular chondrocytes (ACs) in co-culture systems with marrow derived MSCs toward production of more purified cartilage construct. Method: Osteoblasts from rat embryo, chondrocytes from rat knee articular cartilage and MSCs from rat bone marrow were isolated and culture expanded separately into a sufficient numbers required for the following experiments. About 5×106 osteoblasts as well as chondrocytes were then separately suspended in 1 ml alginate solution and the mixture was dropped in calcium chloride solution to form 2-4 mm beads. In parallel passage 3 rat marrow-derived MSCs were cultivated as monolayer in 10cm2 culture dishes. To prepare co-culture systems, the beads consisting of Oss and ACs encapsulated in alginate gel were added to the medium of rat MSCs monolayer cultures and incubated for a period of 60 days. Results: After 1 month, according to scaning electron microscopy (SEM) images, initially fibroblastic feature of co- cultured MSCs, morphologically changed into rather cuboidal cells having the figure of cells involving at active secretary process. Our Real-time PCR analysis, on the other hand, showed the expression of cartilage-specific genes including collagen II and aggreacan in differentiated MSCs, both in ACs and Oss co- culture systems, at the same ratio. Interestingly, based on our findings, the expression of collagen X and osteocalcin genes tended to be significantly down regulated at Oss co- culture system compared to that at ACs co-culture system. Conclusion: These studies revealed that Oss has prominent positive effect on differentiation potential of MSCs toward chondrogenic lineages at a co-culture setup. In this regard it appeared more superior to articular chondrocytes. These results are considered as forward step in therapeutic application of MSCs in defects occurred in articular cartilage and subchondral bone.

Poster Board Number: F-2169

DIFFERENT EXPRESSION PATTERN OF GROWTH FACTORS IN RAT FETUSES WITH SPINA BIFIDA APERTA AFTER *IN UTERO* MESENCHYMAL STEM CELL TRANSPLANTATION

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In a previous study, we investigated prenatal treatment of spina bifida with *in utero* stem cells transplantation in retinoic acid induced spina bifida rat model, our results showed MSCs survived, migrated and differentiated into neural lineage cells. To clarify which growth factor affects the survival, migration and differentiation of MSCs in our model, in this study, we investigated the expression of VEGF-A, FGF4, FGF8, EGF, KGF, IGF, TGF-a, TGF-b, PDGF in rat fetal spinal cord with spina bifida after *in utero* MSCs transplantation. We observed significantly increased expression of EGF and FGF8; expression of FGF4, PDGF and KGF were increased with relatively large internal bias, thus the increasement did not reach statistical significance; while VEGF, TGF-a, TGF-b and IGF expression were not affected. EGF and FGF8 have broad effect on cell proliferation, migration and neurogenesis, our results suggested that intrinsic EGF and FGF8 might play important roles in MSCs survival and migration after *in utero* MSCs transplantation in rat spina bifida model.

Poster Board Number: F-2170

ISOLATION AND CHARACTERIZATION OF NEURONS AND MYOGENIC CELLS FROM RAT MOLAR PERIODONTAL LIGAMENT

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Introduction: The periodontal ligament (PDL) contains various cell populations and plays a central role in maintenance and repair/regeneration of the periodontium, i.e. tooth supporting structures. Because primary cells isolated from PDL tissue are heterogeneous, the development of an effective isolation method for cells of interest is required for application to cell-based therapy. In the present study, we isolated neurons and myogenic cells from primary cultures of rat molar PDL tissue using filter paper for colonial cloning and then characterized these cells by immunocytochemical, RT-PCR and G-banding karyotypic analyses. Materials and Methods: Upper and lower molars were harvested from 6-week-old male SD rats for whole tooth culture. Extracted molars were placed in 60 mm dishes (2-3 teeth per dish) and cultured in DMEM/F12 supplemented with 15% FBS. Fibroblastic and epithelial cells outgrew from PDL tissue surrounding the tooth root and reached confluency within 1.5 months. We found two types of cell populations that showed a small number of long processes or a high cytoplasm to nuclear ratio with some multinucleation. To isolate each cell population, a small piece of filter paper (4 mm2) was soaked with trypsin-EDTA/PBS (-) and then placed onto target cells followed by incubation for 5 min at 37°C. Filter paper containing target cells was placed into a new 60 mm dish and then cultured. Cells outgrew from the filter paper and reached confluency within 2.5 months. Then, cells were subcultured at a 1:3 split ratio. We used cells at passage 4 to identify cell characteristics. Results: After primary cultures reached confluency, we found two types of cell populations. One cell population showed a small number of long processes such as those on

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neurons, and the other cell population was mono and multinuclear with a high cytoplasm to nuclear ratio. These cell types could be subcultured for further analyses. Immunocytochemistry indicated that the cells with long processes were positively stained for nestin, NF-150 and NSE, but were negative for CNPase and GFAP, while the other cell population with mononuclear cells was positive for Myo-D and multinuclear cells were positive for myogenin, myosin and α-SA. RT-PCR showed that cells with long processes expressed nestin, NSE and NF-150, while mono/multinuclear cells expressed Myo-D and myogenin. A proportion of multinuclear cells in primary and subsequent cultures showed spontaneous contraction. Both of these isolated cell populations showed a normal karyotype with a diploid chromosome number. Discussion: Using explant culture of PDL tissue derived from rat molars, we found two morphologically-defined cell populations in primary culture and individually collected cell types using filter paper for colonial cloning of cells for subculture. Based on immunocytochemistry and RT-PCR results, subcultured cells with long processes showed neural cell features without glial cell phenotypes (negative for CNPase and GFAP) and mono/multinuclear cells consisted of myogenic cells, such as myoblasts, myocytes and myotubes, including some cells that showed spontaneous contraction. In addition, isolated cells retained a normal karyotype with a diploid chromosome number. Collectively, these findings suggest the feasibility of obtaining neurons and muscle cells from PDL tissue as an alternative cell source for cell therapies of neural and myodegenerative diseases.

Poster Board Number: F-2171

ACCELERATION OF OSTEOGENIC DIFFERENTIATION OF RAT BONE MARROW MESENCHYMAL STEM CELLS BY CARBON NANOTUBE SCAFFOLDS

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Carbon nanotubes (CNTs) have the desirable mechanical properties for bone substitution materials. To apply CNTs as the material for bone regeneration, we need to evaluate whether CNT scaffolds provide the niches suited for osteogenic differentiation to the residing cells or not. In this study, cover glasses densely coated with single-walled carbon nanotubes (SWNTs) and multi-walled carbon nanotubes (MWNTs) were prepared and evaluated for the osteogenic differentiation of rat bone marrow mesenchymal stem cells (rMSCs) in surface culture. CNT density on glasses was confirmed by the measurement of surface resistivity. Dense coating of SWNTs and MWNTs on cover glasses elevated the expression of runx2 and alkaline phosphatase activity compared to poly-L-ornithine (PLO) coating for cell adhesion. Furthermore, the coating of SWNTs also enhanced the expression of osteocalcin and calcium deposition compared to other samples. Scanning electron microscopic analysis exhibited the needle-shaped crystals deposited on CNTs by mineralization of rMSCs at day 28. In the results, SWNT-coated glasses are superior to PLO-coated and MWNT-coated glasses in promoting bone mineralization.

Poster Board Number: F-2172

ISOLATION AND CHARACTERIZATION OF FELINE ADIPOSE DERIVED MESENCHYMAL STEM CELLS

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Adult stem cells have been used for treating several pathologies. Adipose tissue is an attractive source of mesenchymal stem cells because of its abundance and easy accessibility. In addition, adipose-derived-mesenchymal stem cells from humans, dogs and mice have been shown to possess the capacity to differentiate into several lineages. The main goal of our research was to establish and characterize feline adipose-derived-mesenchymal stem cells (fA-MSC). We isolated and expanded fA-MSC from subcutaneous adipose tissue of 8 cats that had undergone elective ovariohysterectomy. Stem cell isolation was performed using enzymatic digestion (collagenase type I)under gentle agitation at 37°C for 30 minutes. The digested tissues were centrifuged to obtain a pellet. The pellet was ressuspended in Dulbecco's Modified Eagle Medium with 15% fetal bovine serum (FBS), 2mM L-glutamine, 100U/mL penicillin, and 100 mg/mL streptomycin. FA-MSC presented fibroblast-like morphology. Cells were positive for CD44; CD90, Stro-1 and Nanog using flow citometry analysis, and were negative for CD45 and HLA-DR. The fA-MSC were able to show osteocytic phenotypes when exposed to appropriate induction media, based on cell morphology. Four nude mice were taken to the tumorigenicity potential assay. Cells were injected intramuscular and the animals were observed for 3 months. After that the animals were sacrificed by under deep anesthesia. Histopathological examinations were performed on the muscle transplantation site, also in heart, lungs, liver, spleen, kidneys and brain. After fixation in 10% neutral-buffered formalin, all tissues were processed in a routine manner, embedded in paraffin, sectioned, and stained with HE for light microscopic evaluation, and none of them showed any changes in morphology. Thus, in our knowledge, this is the first work with fA-MSC, which, according to our results, seems to represent a promising type of progenitor cell for autologous cellular-based therapies in veterinary medicine. Therefore the characterization of feline mesenchymal stem cells will facilitate future studies of stem cell biology and therapeutics for the domestic cat.

Poster Board Number: F-2173

CHARACTERIZATION AND DIFFERENTIATION OF MESENCHYMAL STEM CELLS ISOLATED FROM CANINE SYNOVIAL FLUID

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Aging-related articular chondrogenic diseases are higher in aged female and chondrocyte is a good candidate for replacement by an engineered tissue using mesenchymal stem cells (MSCs). Gender differences in MSCs suggest that the age-and gender-related articular chondrogenic diseases may involve in stem cell loss and differentiation potential. The synovial tissues are a valuable MSCs source for cartilage tissue engineering because these cells are easily obtainable by the intra-articular biopsy during diagnosis. In this study, we isolated and characterized the canine MSCs derived from synovial fluid (cSF-MSCs) of female and male donors. Synovial fluid was flushed with saline solution from post-puberty female and

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male dogs, and cells were isolated and cultured in advanced-DMEM (A-DMEM) supplemented with 10% FBS in a humidified 5% CO_2 atmosphere at 38.5°C. The cells were evaluated for the expression of MSC specific markers, such as CD29, CD44 and CD90 using flow cytometry, and expression of the early transcriptional factors, such as Oct3/4, Nanog and Sox2 by RT-PCR. The cells were induced under conditions conductive for adipogenic, osteogenic, and chondrogenic lineages, then evaluated by specific staining (Oil red O, von Kossa, and Alcian Blue staining, respectively) and analyzed for lineage specific markers by RT-PCR. Female and male cSF-MSCs were positive for alkaline phosphatase activity and expressed MSC specific markers (CD29, CD44 and CD90). Early transcriptional factor (Oct3/4, Nanog and Sox2) were also positively detected in both cSF-MSCs. Further, these MSCs were observed to differentiate into mesenchymal lineages, such as adipocytes (Oil red O staining), osteocytes (von Kossa staining), and chondrocytes (Alcian Blue staining) by cell specific staining. Lineage-specific genes (osteocyte; osteonectin and Runx2, adipocytes; aP2 and PRAR-γ2, and chondrocytes; aggrecan and collagen type-2) were also detected in both cSF-MSCs. In this study, we successfully established synovial fluid derived MSCs from female and male dogs, and determined their basic biological properties and differentiation ability. These results suggested that synovial fluid is a valuable MSC source for cartilage regeneration therapy, and it is easily accessible from osteoarthritic knee. Further studies are being conducted to standardize the optimal parameters for efficient differentiation of SF-MSCs into chondrocytes.

Poster Board Number: F-2174

TRANSPLANTATION OF ADIPOSE TISSUE DERIVED MULTI LINEAGE PROGENITOR CELLS REDUCES SERUM CHOLESTEROL IN HYPERLIPIDEMIC WATANABE RABBITS

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Background: Familial hypercholesterolemia (FH) is an autosomal co-dominant disease characterized by high concentrations of pro-atherogenic lipoproteins and premature atherosclerosis. We examined the response to *in situ* stem cell therapy using adipose tissue-derived multi-lineage progenitor stem cells (hADMPC) in the LDL-receptor deficient Watanabe heritable hyperlipidemic (WHHL) rabbit, an animal model for homozygous FH. Methods: WHHL rabbits received either normal control rabbit-derived, GFP-rabbit-derived or WHHL rabbit-derived ADMPC (normal-ADMPC, GFP-ADMPC and diseased-ADMPC, respectively) via the portal vein. This was followed by 12-week immunosuppressive therapy to avoid allogenic rejection. *In situ* survival and differentiation of the ADMPC into hepatocytes was examined by flow cytometry and immunohistochemical analysis, respectively. Lipid profile was examined before-, and 4-, 8- and 12 weeks after transplantation. LDL clearance was examined at the end of the study by125I-LDL turnover. Results: *In situ* survival of GFP-ADMPC was confirmed after transplantation. The cells integrated into the hepatic parenchyma and co-expressed GFP and hepatocyte markers such as albumin, indicating that the cells were reprogrammed into hepatocytes-like cells *in situ*. Transplantation of normal-ADMPC but not diseased-ADMPC resulted in a significant reduction of serum total- and LDL- cholesterol after transplantation. 125I-LDL turnover study showed significant improvement in the rate of LDL clearance in the WHHL rabbits with transplanted normal-ADMPC but not in those transplanted with diseased-ADMPC. Conclusion: Transplantation of ADMPC but not diseased ones corrected the metabolic defects in WHHL rabbits, suggesting that ADMPC transplantation is a potentially useful therapy for FH.

Poster Board Number: F-2175

COMPARISON OF *IN VITRO* HEPATOGENIC DIFFERENTIATION POTENTIAL BETWEEN VARIOUS PLACENTA-DERIVED STEM CELLS AND OTHER ADULT STEM CELLS AS AN ALTERNATIVE SOURCE OF FUNCTIONAL HEPATOCYTES

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Mesenchymal stem cells (MSCs) are powerful sources for cell therapy in regenerative medicine. The capability to obtain effective stem cell-derived hepatocytes would improve cell therapy for liver diseases. Recently, various placenta-derived stem cells (PDSCs) depending on the localization of placenta have been suggested as alternative sources of stem cells are similar to bone marrow-derived MSC (BM-MSCs) and adipose-derived MSC (AD-MSCs). However, comparative studies for the potentials of the hepatogenic differentiation among various MSCs largely lacking. Therefore, we investigated to compare the potentials for hepatogenic differentiation of PDSCs with BM-MSCs, AD-MSCs, and UCB-MSCs. Several MSCs were isolated from human term placenta, adipose tissue, and umbilical cord blood and characterized isolated MSCs and BM-MSCs was performed by quantitative reverse transcription-PCR (RT-PCR) and special stains after mesodermal differentiation. The hepatogenic potential of PDSCs was compared with AD-MSCs, UCB-MSCs, and BM-MSCs using RT-PCR, PAS stain, ICG up-take assays, albumin expression, urea production, and cytokine assays. MSCs isolated from different tissues all presented similar characteristics of MSCs. However, the proliferative potential of PDSCs and the expression of hepatogenic markers in differentiated PDSCs were higher than other MSCs. Interestingly, the expression of hepatocyte growth factor (HGF) increased in PDSCs after hepatogenic differentiation. Interestingly, stem cell factor (SCF) expression in chorionic plate-derived MSCs, one of the PDSCs, was significantly higher than in the other PDSCs. Taken together, the results of the present study suggest that MSCs isolated from various adult tissues can be induced to undergo hepatogenic differentiation *in vitro*, and that PDSCs may have the greatest potential for hepatogenic differentiation and proliferation. Therefore, PDSCs could be used as a stem cell source for cell therapy in liver diseases.

Poster Board Number: F-2176

INFLUENCE OF DONOR AGE ON THE DIFFERENTIATION OF PORCINE MESENCHYMAL STEM CELLS INTO MYOGENIC CELL LINEAGES

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The use of porcine mesenchymal stem cells (MSCs) has become highly relevant in preclinical assessment of therapeutic modalities

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for regenerative medicine and tissue engineering applications. We have previously shown donor age related differences in proliferation rate, colony-forming ability and osteogenic differentiation capacity of porcine MSCs. In this study, we compared the myogenic differentiation potential of MSCs derived from bone marrow extract of postnatal (~2 weeks old) and adult (~8 months old) domestic pigs. Isolated MSCs (2-5 passages) were induced into smooth muscle cells (SMCs), skeletal muscle cells (SkMCs) and cardiomyocyte-like cells (CMCs) following the protocols described previously. Induced MSCs were observed for the morphological changes, and analyzed the expression of myocyte specific markers by reverse transcription-polymerase chain reaction (RT-PCR), quantitative PCR (qPCR), immunofluorescence staining and western blotting to examine the rate of differentiation at the mRNA and protein levels. Upon induction in each specific media, MSCs from postnatal and adult donors showed spindle morphology with intracellular thin filaments resembling SMCs, and generated myotube-like structures reminiscent of skeletal muscle phenotype. Similarly, morphology of cells after cardiomyogenic induction was changed and stick-like cells were prominently seen along with a few cell aggregates. Time-course analysis on the differentiation of MSCs into muscle-lineage cells by RT-PCR and qPCR demonstrated the detection and up-regulation of markers specific to SMCs: alpha-smooth muscle actin (α-SMA), calponin (CNN), smooth muscle 22 alpha (SM22α) and smooth muscle-myosin heavy chain (SM-MHC); SkMCs: MyoD, myogenin, desmin and actin, alpha 1, skeletal muscle (ACTA1); and CMCs: α-SMA, desmin, cardiac troponin T (CTT) and cardiac α-actin. The positive expression of these lineage specific markers was further revealed by immunofluorescence and western blot analyses. Importantly, the quantitative expression of markers at both the mRNA and protein levels in differentiated muscle lineage cells was higher in MSCs from postnatal donor than those from adult donor. In conclusion, the findings of this study show that the myogenic differentiation potential of porcine MSCs tends to decrease with increasing age of the donor. In this context, porcine MSCs of postnatal origin seem to be an ideal source to examine the therapeutic applications using pig as a suitable animal model. This work was supported by Basic Science Research Program through the National Research Foundation (NRF) funded by the Ministry of Education, Science and Technology (2010-0010528) and BioGreen 21, Rural Development Administration (20110701-305-533-001-02-00), Republic of Korea.

Poster Board Number: F-2178

A 3-DIMENSIONAL CULTURE SYSTEM FOR ENHANCED DIFFERENTIATION OF MESENCHYMAL STEM CELLS INTO THE CARDIAC LINEAGE

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Inadequacies in current treatment options for severe heart failure have shifted the focus on to promising alternatives such as cell-based therapies for the prevention or treatment of cardiac dysfunction. Bone marrow derived Mesenchymal Stem Cells (MSCs) have been shown to differentiate into cardiomyocytes *in vitro* through techniques that include co-culture and chemical stimulation by 5-Azacytidine. The efficiency of differentiation and ease in harvesting of these differentiated MSCs can be improved by three-dimensional (3D) culture systems as cell-cell interaction has been shown to be important for bone marrow stromal cells to differentiate into cardiomyocytes. We developed a 3D culture technique which forced murine bone marrow derived MSCs to form aggregates which were maintained in suspension cultures. The suspension cultures and conventional monolayer cultures were subjected to adipogenic (using Indomethacin, IBMX and dexamethasone) and osteogenic (using β-glycerophosphate, ascorbic acid and dexamethasone) stimuli. Adipogenic and osteogenic differentiation was enhanced in 3D cultures with increased triglyceride accumulation and mineralization respectively. This 3D culture based differentiation technique also generates aggregates that can be easily harvested following differentiation. When shifted to conventional monolayer culture, these 3D aggregates of MSC assumed the morphology typical of adherent monolayers and continued to differentiate much more efficiently than the standard monolayer culture. Cardiomyogenic differentiation in monolayer cultures of MSC was standardized for early passages (1-8) using 10 μM 5-Azacytidine and assayed for cardiac specific markers using qPCR which indicated increased expression of GATA-4, Nkx2-5 and Mef2c by 1.8, 3.9 and 6.1 fold respectively. The application of 5-azacytidine as described above, in 3D cultures of murine and human MSCs may yield much more efficient differentiation into the cardiomyogenic lineage as was confirmed by the adipogenic and osteogenic experiments.

Poster Board Number: F-2179

APPLICATION OF IPS CELLS TO THE RESEARCH OF FIBRODYSPLASIA OSSIFICANS PROGRESSIVA

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Fibrodysplasia Ossificans Progressiva (FOP) is a rare genetic disease characterized by progressive ectopic ossification, which severely inhibits patients' ADL. The responsible gene for FOP is the ALK2 (activin like kinase 2) gene, which is one of type I receptors for BMP. Mutations found in patients transform ALK2 protein into a constitutive active form, which transduces the BMP signal without a ligand binding. This causes ectopic ossification in muscles, tendons, and ligaments, although precise mechanisms are not yet known. Harvesting target tissues from patients is strictly prohibited because tissue damage accelerates the ectopic ossification. This issue now can be overcome by using iPSCs derived from patients. We have established iPSCs from patients with FOP, from which cells in mesenchymal lineages are induced such as bone, cartilage, muscle, or tendon cells. We are currently analyzing the difference between wild type iPSCs and FOP-iPSCs during osteogenesis and chondrogenesis. We also try to recapitulate disease's phenotype *in vitro* by the stimulation with factors related to tissue damage. In this presentation, we will report our recent data and discuss the possibility of drug discovery by using our system.

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Poster Board Number: F-2180

EXTRACELLULAR MATRIX INDUCE THE ENDOTHELIAL POTENTIAL OF BONE- DERIVED MESENCHYMAL STEM CELLS (MSCS)

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Bone is a highly vascularized tissue and previous studies have demonstrated that interactions between blood vessels and osteoblasts (OBs)_bone-forming cells_are critical for successful bone development. OBs are known to play a important role in maintaining one of the hematopoietic niches in the bone marrow (BM) along with a number of other non-hematopoietic cells (NHCs), including endothelial cells (ECs) and mesenchymal stem cells (MSCs)_multilineage adult somatic stem cells which have been isolated from the BM and other tissue, including bone and fat. Currently, the exact role of each NHC in the BM, their identity, and differentiation capacity are being intensely studied. Thus, we investigated the differentiation capacity of MSCs derived from BM and bone, including pre-OBs and trabecular bone-derived MSCs. We found that MSCs derived from bone tissue express similar surface markers as BM MSCs, and can also differentiate into tri-mesodermal lineages of bone, fat, and cartilage. However, under EC differentiation conditions, only bone-derived MSCs (B-MSCs) can form tubular structures and uptake low-density lipoproteins (LDL), fulfilling the functional criteria for ECs. When cultured in EC conditions, B-MSCs show protein expression of a number of EC markers, including CD31/PECAM, Flk-1, V-CAM, and von Willebrand Factor. Moreover, in the *in vivo* chick chorioallantoic membrane assay, these B-MSCs significantly enhance vessel formation. Mechanistically, the endothelial differentiation capacity of B-MSCs may be due to the activation of the endothelial transcription factor forkhead box protein C2 (FOXC2) by the extracellular matrix (ECM), an important component in endothelial differentiation. Increased expression of FOXC2 was found in B-MSCs but not BM MSCs after culturing in ECM and tube formation induction, and this was associated with upregulation of CD61/integrin αvβ5, a receptor for many ECM proteins and a critical factor in angiogenesis. Knockdown of FOXC2 in B-MSCs by RNA interference resulted in a significant decrease of CD61 expression. Taken together, our findings reveal the endothelial potential of B-MSCs, and suggest a role for the ECM in mediating B-MSC lineage commitment towards an endothelial phenotype.

Poster Board Number: F-2181

THE EFFECT OF DBP/PLGA SCAFFOLDS FOR DIFFERENTIATION AND PROLIFERATION OF INTERVERTEBRAL DISC DERIVED FROM MESENCHYMAL STEM CELLS

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Demineralized bone particle (DBP) that affects to cell proliferation and differentiation has been used as biomaterials. PLGA (poly(lactic-co-glycolic acid) nanofiber scaffolds accommodate continuous differentiation of mesenchymal stem cells (MSCs) into osteoblastes and chondrocytes. We fabricated MSCs seeded on DBP/PLGA scaffolds with various content of DBP (20, 40 and 80%) and pure PLGA scaffolds. MSCs seeded on DBP/PLGA scaffold into intervertebral discs in *in vivo* culture and we studied RT-PCR, MTT and histological assay. In this study, we confirmed that MSCs were differentiated into Intervertebral Discs in the DBP/PLGA scaffolds through the histological assay. MTT results, cell proliferation was good in DBP/PLGA scaffolds. The RT-PCR results showed specific mRNA expression. In conclusion, this study suggested that the scaffolds of higher contents of DBP were good results of proliferation, inflammation and differentiation. This research was supported by WCU (R31-20029), SCRC (SC4110) and MBC (0405-BO01-0204-0006)

Poster Board Number: F-2182

UNDERSTANDING THE PATHOLOGY OF THE ARTHROPATHY IN CHRONIC INFANTILE NEUROLOGICAL CUTANEOUS AND ARTICULAR SYNDROME BY USING IPS CELLS TECHNOLOGY

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Patients of chronic infantile neurological cutaneous and articular (CINCA) syndrome demonstrate nonpruritic uriticarial rash from birth, central nervous system disorders including meningitis and deafness, and articular symptom. The syndrome is caused by heterozygous mutation of NLRP3 gene and gain of function mutations of NLRP3 causes excessive production of IL1 beta. Mutant NLRP3 protein in macrophages causes overexpression of IL1 beta, and IL1-blocking agent is now in clinical use and successfully improves the inflammatory symptoms. Although cartilage cells express NLRP3 as well as macrophages, IL1-blocking agent is not effective for arthrophathy suggesting alternative pathological processes responsible for arthropahty. X-ray findings of involved long bones show enlargement of epi-metaphyseal portion with abnormal intramedurally calcification, and histological analyses demonstrate complete disorganization of growth plate cartilage without infiltration of inflammatory cells. These findings suggest that primary foci for this pathological condition are growth plate cartilage cells, which are, however, difficult to be obtained from patients. Therefore we take the approach to use induced pluripotent stem cells (iPSCs) derived from CINCA patients, recapitulate the pathology *in vitro*, and study the mechanisms of arthropathy. We have established iPSCs with and without a mutant NLRP3 gene from CINCA patients, differentiated them into cartilaginous linage, and are analyzing the phenotypic and genotypic difference between them, which we hope leads to the understating the pathology and developing treatment for arthropathy.

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Poster Board Number: F-2183

RECONSTITUTION OF ARTICULAR (JOINT) CARTILAGE DEFECTS BY AURICLE (EAR) DERIVED STEM/PROGENITOR CELLS

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Objective:Due to limited regenerative capacity of damaged articular cartilage, joint surface injuries regularly progress to more serious disorders, such as osteoarthritis. Current surgical interventions fail to reconstruct hyaline cartilage, therefore cartilage defects are a primary target for regenerative medicine. We recently identified a promising cartilage stem/progenitor cells (CSPCs) from human auricular (ear) perichondrium towards regenerative medicine of elastic cartilage such as auricle. However, the differentiation potential of CSPCs for hyaline cartilage, remains to be elucidated. Using canine models, we here examined whether CSPCs differentiate into hyaline cartilage tissue without detectable elastic fibers, and to reconstruct full-thickness articular (joint) cartilage defects in a canine model. Methods:CSPCs were harvested from auricular (ear) cartilage perichondrium of TOYO beagle. To characterize the canine CSPCs, colony-forming assays, multiple differentiation assay and autologous transplantation into full-thickness cartilage defects were performed. The transplanted cells were assessed at 2 months post-transplantation by the International Cartilage Repair Society (ICRS) histological scoring system and imunohistochemistry analysis. Results:Canine CSPCs possessed the highest proliferative capacity than auricular chondrocytes (AuCs) and articular chondrocytes (ArCs), CSPCs: 9.22 colonies (SD±3.15), AuCs: 3.11 colonies (SD±1.29), ArCs: 2.00 colonies (SD±1.97). Canine CSPCs are not able to differentiate into multiple lineages. Transplanted cells differentiated into hyaline like cartilage forming extracellular matrix that displayed positive for collagen type II and negative for elastin. ICRS histological scoring of the reconstruction was 12 points, which was comparable to normal tissues. Conclusion:We showed that auricle (ear)-derived CSPCs are capable of differentiating into mature chondrocytes of hyaline cartilage without detectable elastic fibers, resulting in reconstruction of full-thickness articular (joint) cartilage defects. Utilization of CSPCs will provide a promising cell source for treating articular (joint) cartilage defects with minimally invasive approach.

Poster Board Number: F-2184

EFFECT OF UNIAXIAL CYCLIC STRAIN ON MYOGENESIS OF ADIPOSE-DERIVED STEM CELLS

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Adipose-derived stem cells (ASCs) hold potential for engineering of muscle tissue due to their ability to differentiate into the skeletal myogenic lineage. Current approaches to induce *in vitro* skeletal muscle differentiation require long culturing time and result in a poor yield. Previously, we have established the role of uniaxial cyclic tensile strain (CTS) in driving the assembly and differentiation of skeletal myocytes *in vitro* using mouse model system. The aim of the current work was to explore the effect of CTS on the skeletal myogenic differentiation of ASCs.ASCs were cultured on flexible-bottomed culture plates (Flexcell). After confluence, the cells were incubated in reduced serum conditions and subjected to uniaxial CTS for 48 h. Differentiation profiles were assessed by morphological and biomolecular indicators.The CTS protocol produced an array of cells aligned perpendicularly to the axis of strain. In addition, mechanical stimulation induced a significant increase in the number of multinucleated cells. Immunofluorescence staining revealed the presence of a large percentage of myosin heavy chain positive myotubes. Furthermore, the myofibrillogenesis was confirmed by the presence of actin/myosin cross striations.These results indicate that mechanical stimulation increases the rate of myogenic differentiation of ASCs *in vitro*. This opens the perspective of using mechanically preconditioned ASCs for tissue engineering applications involving muscle tissue.

Poster Board Number: F-2185

GENERATION OF OLIGODENDROCYTE PRECURSORS FROM BONE MARROW STROMAL CELLS FOR REMYELINATION THERAPY

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CNS injury induces demyelination and severe loss of function. We hypothesize that transplantation of adult bone marrow-derived oligodendrocyte precursor cells (OPCs) provides for remyelination during tissue repair. We attempted therefore to direct differentiation of bone marrow stromal cells (BMSCs, adult rats) along the oligodendroglial lineage in vitro. Successful differentiation was achieved by a novel protocol. BMSCs were first cultured as non-adherent spheres until they expressed markers of neural/glial progenitors (BM-NGPs). These BM-NGPs were then maintained in adherent culture supplemented with β-heregulin (β-Her), PDGF-AA and bFGF. Oligodendrocyte precursors expressing OPC markers - NG2, Olig2, PDGFRa and Sox10, were generated within two weeks and can be expanded in culture for up to 3 months with no observable decline in OPC marker expression. We further performed both in vitro and *in vivo* assays to analysis the myelination capability of these BMSC-derived OPCs (BM-OPCs), BM-OPCs co-cultured with dorsal root ganglion neurons for 2 weeks extended myelin basic protein (MBP)-immunopositive processes along neuritis, suggesting maturation into myelinating oligodendrocytes. BM-OPCs were also injected into the retina of adult rats, where naturally unmyelinated axons can be used as a platform for study of *in vivo* myelination. MBP-immunopositive processes were also observable along retinal axons by 8 weeks post-injection. Our findings indicate BMSCs as a possible source of OPCs for autologous transplantation. The results also support the possibility of directed differentiation BMSCs along specific neural/glial lineages.

Poster Board Number: F-2186

IN VIVO TRACKING OF MESECHYMAL STEM CELLS USING FLUORESCENT NANOPARTICLES IN AN OSTEOCHONDRAL REPAIR MODEL

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We devised and tested an *in vivo* system to monitor the migration of mesenchymal stem cells (MSCs) within the marrow cavity. In vitro studies confirmed that PDGF-AA had the most potent chemotactic effect of the tested factors, and possessed the greatest number of receptors in MSCs. MSCs were labeled with fluorescent nanopar-

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ticles and injected into the marrow cavity of nude rats through osteochondral defects created in the distal femur. The defects were sealed with HCF (heparin-conjugated fibrin) or PDGF (platelet-derived growth factor)-AA-loaded HCF. In the HCF-only group, the nanoparticle-labeled MSCs dispersed outside the marrow cavity within 3 days after injection. In the PDGF-AA-loaded HCF group, the labeled cells moved time-dependently for 14 days toward the osteochondral defect. HCF-PDGF in low dose (8.5 ng/μl) was more effective than HCF-PDGF in high dose (17 ng/μl) in recruiting the MSCs to the osteochondral defect. After 21 days, the defects treated with PDGF and TGF-β1-loaded HCF showed excellent cartilage repair compared with other groups. Further studies confirmed that this *in vivo* osteochondral MSCs tracking system (IOMTS) worked for other chemoattractants (CCL-2 and PDGF-BB). IOMTS can provide a useful tool to examine the effect of growth factors or chemokines on endogenous cartilage repair.

Chromatin in Stem Cells

Poster Board Number: F-2187

ESTABLISHMENT OF IPS CELL LINES FROM THE FOUNDER TRAINS OF THE HXB/BXH RAT RI PANEL TO STUDY THE EFFECTS OF STRUCTURAL VARIATIONS ON MOLECULAR PHENOTYPES

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Structural variations (SVs) are genomic rearrangements of >50 bp in length and comprise deletions, insertions, inversions, transpositions of mobile elements, duplications, and translocations. Although SVs are the main sources of genetic diversity, the effects of SVs on genome architecture, nucleosome positioning, epigenetic modifications, and gene expression are largely unknown. The extensively studied recombinant inbred (RI) rat panel HXB/BXH provides an ideal model system for genome-wide and targeted techniques to understand the impact of SVs on quantitative and qualitative gene expression characteristics. We have derived induced pluripotent stem (iPS) cell lines from the founder strains (SHR and BN-Lx) of the RI panel using a multicistronic reprogramming vector containing the four Yamanaka-factors. We examined the karyotypes of these cell lines and their ability to differentiate into derivatives of the three germ layers. The potential of these iPS cell lines to efficiently differentiate into a variety of specialized cell types (e.g. neurons) enable us to examine the effects of SVs on molecular phenotypes in different contexts. To functionally validate observed correlations between SVs and molecular phenotypes, we have designed TAL-effector nucleases (TALENs) in the proximity of SVs. These TALENs will be used in combination with homologous DNA to target the SV in iPS cells. In this way, we attempt to exchange SVs between strains as a functional validation.

Poster Board Number: F-2188

THE IMPACT OF EPIGENETIC MODIFICATION ON THE HETEROGENEITY AND DIVISION SYMMETRY OF MURINE ES CELLS THROUGH SINGLE-CELL RNA ANALYSES

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It is widely known that certain types of cancer are initially sensitive to drug treatment, but eventually become resistant to therapeutic interventions as the disease progresses. This phenomenon can be explained by the presence of population heterogeneity within tumours, since cancer cell populations are indeed associated with both genetic and epigenetic instability. Such population heterogeneity would allow the development of subpopulations with genetic and epigenetic changes that would give some cells selective growth advantages, potentially leading to drug and chemo resistance. Accumulating evidence underscores the importance of studying how clonal heterogeneity is generated by epigenetic mechanisms. In this project we will use murine ES cells as a model system to understand the fundamental mechanisms in the generation of clonal heterogeneity in a given cell population. Epigenetic information is transmitted through chemical modification of chromatin components, and the fidelity of inheritance during DNA replication is believed to be precise. High-fidelity inheritance is believed to be essential for the maintenance of lineage identity, because error-prone inheritance could result in distinct epigenetic modification states between sister chromatids, each of which will be independently delivered to each daughter cell to generate a distinct phenotype. On the other hand, indirect evidence suggests that the fidelity of epigenetic inheritance could be lower than previously thought. Our hypothesis is that clonal heterogeneity and asymmetric divisions, both of which are observed in various lineages, are in part driven by error-prone epigenetic inheritance. In this context, using murine ES cells we examined the levels of clonal heterogeneity and symmetry of daughter cells through a high-throughput single cell RNA analysis via the Biomark platform. Our data from single-cell quantitative RNA analyses using pairs of daughter cells suggest that while Oct4 expression tends to be similar between daughter cells, half of the pairs of daughter cells displayed an asymmetric expression pattern for a subset of genes (e.g. Nanog). Furthermore, by analysing mutant cells that lack chromatin modifiers, and wild type cells treated with epidrugs we evaluated whether deregulation of the epigenetic system could generate phenotypic variations within a population. Our experiments will help determine what kind of intrinsic signals are involved in the generation of clonal heterogeneity.

Poster Board Number: F-2189

THE HISTONE DEACETYLASE SIRT6 MODULATES MOUSE EMBRYONIC STEM CELL DIFFERENTIATION

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Embryonic Stem (ES) cells have the ability to proliferate indefinitely and can differentiate into all derivatives of the three primary germ layers such as ectoderm, mesoderm and endoderm. We found that the mammalian histone deacetylase SIRT6 plays a critical role in embryogenesis. Upon differentiation, ES as well as iPS cells derived from SIRT6 deficient mice generate embryoid bodies (EBs) with underdeveloped endoderm and mesoderm, but overdeveloped neuroectoderm. This phenotype is associated with a decreased expression of Gata4, Afp, Hnf4, Lamb1, Brachyury and Kdr, which are critical for endoderm and mesoderm development. Elevated levels of Fgf5, Nestin and GFAP correlate with the preferential neuroectoderm in EBs lacking SIRT6. We found the expression of OCT4, SOX2 and NANOG to be upregulated in EBs from SIRT6 knockout mice. Consistently, we observed increased acetylation of H3K56 in the promoters of these pluripotent genes, indicating that SIRT6 functions as an histone deacetylase to repress pluripotent genes during early differentiation. Additionally, EBs lacking SIRT6 exhibit a significant increment in the expression of a FoxP1-ES splice variant

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shown to be required for the transcriptional activation of pluripotent genes including OCT4 and NANOG. Furthermore, we find bulk genomic levels of 5hmC to be increased in SIRT6 deficient ES cells, which correlate with increased levels of the hydroximethylase Tet2. Interestingly, it has been shown that OCT4 positively regulates the expression of Tet1 (Koh et al., 2011). Overall, we proposed a model where the deacetylase activity of SIRT6 causes the transcriptional repression of OCT4, which in turn regulates the expression of Tet1 and possibly Tet2 as an epistatic pathway required for proper embryogenesis. This model also includes the ability of SIRT6 to repress the expression of the Foxp1-ES splice variant required for maintaining pluripotency by promoting expression of OCT4 and NANOG. Thus, SIRT6 plays a critical role during embryonic differentiation as a modulator of an epigenetic cross-talk that includes: 1) repression of pluripotency by deacetylation of H3K56 on the core pluripotent genes OCT4, SOX2 and NANOG; 2) repression of Tet1 and Tet2 expression via regulation of OCT4 levels; and 3) repression of FoxP1-ES splice variant as a mean to further inhibit transcriptional expression of OCT4 and NANOG.

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MICRORNA-323-3P-DEPENDENT REGULATION OF THE POLYCOMB PROTEIN EED IN MOUSE EMBRYONIC STEM CELLS<!--ENDFRAGMENT-->

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The Polycomb Repressive Complex 2 (PRC2) mediates epigenetic gene silencing by trimethylating histone H3 lysine 27 (H3K27me3) and exerts essential functions in many fundamental biological processes. It is believed that PRC2 is targeted to chromatin by the Eed protein subunit to methylate H3K27, leading to a repressive chromatin state that inhibits gene expression. MiRNAs are a class of ~22 nt non-coding RNA molecules that modulate gene expression at the post transcriptional level by sequence complementarity. Our previous study has shown that the expression of miRNA clusters located in the imprinted Dlk1-Dio3 region on mouse chromosome 12 correlates with the developmental potential of mouse embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). MiR-323-3p is an miRNA of the Dlk1-Dio3 region with high expression in mouse ESCs and fully pluripotent iPSCs. Bioinformatic prediction identifies two putative miR-323-3p binding sites in the 3′untranslated region (3′UTR) of Eed mRNA, suggested that the expression of Eed may be regulated by miR-323-3p. Real-time PCR and western blot showed that both the mRNA and protein levels of Eed were lower in mouse embryonic fibroblast (MEF) cells as compared to mESCs, in which the endogenous miR-323-3p expression was much higher than that in mESCs. Over expression of exogenous miR-323-3p was able to suppress Eed mRNA expression and resulted in decreased Eed protein level in mESCs. On the contrary, Eed mRNA and protein levels increased when mESCs were transfected with miR-323-3p inhibitor. Luciferase assays proved that mutations in both the miR-323-3p binding sites in Eed 3′UTR abolished the inhibitory effects of miR-323-3p on Eed, confirmed their target relationship. The reduced Eed expression was able to affect PRC2 functions as evidenced by the significantly reduced H3K27me3 level in MEF as compared to mESCs. Overexpression of miR-323-3p in mESCs resulted in decreased H3K27me3 level, whereas the effect was rescued by the addition of miR-323-3p inhibitor. In conclusion, we demonstrate that miR-323-3p is differentially expressed in MEF and mES cells. One function of miR-323-3p is to regulate the PRC2 complex by targeting to the 3′UTR of Eed mRNA, and such regulation will result in altered H3K27me3 level in MEF and mESCs. <!--EndFragment-->

Poster Board Number: F-2191

LIVE CELL IMAGING OF XIC PAIRING IN DIFFERENTIATING ES CELLS AND THE ESTABLISHMENT OF ALLELIC ASYMMETRY DURING RANDOM X INACTIVATION

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Random X inactivation represents a paradigm for monoallelic gene regulation and epigenetic changes during early ES cell differentiation. The choice of X chromosome to inactivate in XX cells is ensured by monoallelic regulation of Xist RNA via its antisense transcription unit Tsix/Xite. Homologous pairing events have been proposed to underlie asymmetric Tsix expression but this has never been addressed experimentally, owing to the dynamic and transient nature of such early developmental events. Here we investigate the live cell dynamics and the outcome of Tsix pairing in differentiating mouse ES cells. We find an overall increase in genome dynamics, including the Xics, during early ES cell differentiation. When they become paired however, Xic loci show markedly reduced movements. Upon separation, Tsix expression becomes transiently monoallelic, thus providing a window of opportunity for Xist up-regulation in cis to the silent Tsix allele. Our findings reveal the spatio-temporal choreography of the two X chromosomes during early differentiation, and point to a direct role for pairing events in facilitating symmetry-breaking and monoallelic regulation of Xist during random X inactivation.

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PDGF SIGNALING REGULATES THE CHROMATIN LANDSCAPE OF ADULT CARDIAC STEM CELLS

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The discovery of multipotent cardiac stem cells suggests a reserve capacity for cardiac self-renewal during homeostasis and potentially regeneration. Our studies focus on a novel population of Sca1 and Pdgfrα-positive adult cardiac-resident MSC-like stem cells in the mouse heart, which can self-renew and differentiate into cardiomyocytes, smooth muscle cells and endothelial cells. The Pdgfrα-expressing population captures all CFU-F (colony forming units - fibroblast) ability *in vivo*. PDGF appears to play a key role in the activation, maintenance and restoration of the stem cell state in the cardiac CFU-F cells, while inhibition of PDGF signaling induces a state of cellular quiescence. The capacity of stem cells to switch between quiescence and self-renewal while maintaining multipotency for lineage-specific differentiation is likely governed by epigenetic mechanisms, including DNA methylation and histone modifications. We have delineated the chromatin landscape of cardiac CFU-F cells using ChIP-on-chip analysis. Murine cCFUF-Fs (Pdgfrα+ Sca1+ Pecam1-) were isolated from the adult heart and cultured in the presence and absence of either PDGF ligand or inhibitor. ChIP analysis of activating and repressive histone modifications using whole genome promoter arrays identified the regulato-

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ry epigenetic networks associated with active and inhibited PDGF signaling. Our analysis reveals markedly different chromatin states in these stem cells in the context of PDGF stimulation and inhibition. Distinct promoters (representing distinct cellular processes) were found to be enriched with H3K4me3 (active) and H3K27me3 (repressed) marks, in PDGF ligand-treated vs PDGF inhibitor-treated cells. Most interestingly, in these adult progenitors, we find evidence for bivalent domains, many of which are associated with known cell fate regulatory genes that are bivalently marked in embryonic stem cells. Our data indicate that PDGF signaling may assert an epigenetic memory in cCFUFs and suggest a key role for Polycomb mediated regulation in adult stem cells.

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LONG DISTANCE INTERACTION OF 4Q35 LOCUS: THE THREE DIMENSIONS OF THE FACIO-SCAPULO-HUMERAL DYSTROPHY.

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Facio-scapulo-humeral dystrophy (FSHD) is an autosomal dominant, neuromuscular disorder, characterized by facial muscular weakness with progression to the rest of the body. Asymmetric involvement of affected musculature is highly characteristic of FSHD. Linkage analyses have shown that 95% of cases are associated with D4Z4 deletion, a 3.3 kb tandemly repeated sequence, which is located in the subtelomeric region of the long arm of chromosome 4, 4q35. However, no candidate gene has been clearly identified, and families with typical FSHD phenotype, but no linkage to 4q35 (FSHD2) suggest complex epigenetic mechanisms. The deletion of repetitive elements and changes in the epigenetic marks across the D4Z4 array such as DNA hypomethylation or decrease in H3K9 trimethylation also indicates that FSHD involves changes in chromatin organization and epigenetic alterations. Gene localization in the nucleus is not arbitrary, it's a dynamic process and we have previously identified D4Z4 as the first human sequence able to control the localization of its abutting telomere by tethering this chromosome end toward the nuclear periphery. Furthermore, the localization of chromosome ends modifies the timing of replication suggesting that We hypothesize that the tridimensional organization of the 4q35 locus is altered in patients, being responsible for deregulation of disease's gene expression. The aims of our study are to compare chromatin conformation of the 4q35 locus in patients and unaffected individuals, and what are the interactions between sequences, in close proximity or distant, in the FSHD pathogenesis during proliferation of muscle precursor cells and muscle differentiation. Thus, we have developed a FISH technique coupled with immunofluorescence in 3D in order to study the tridimensional distribution of DNA sequences within the nuclear volume. Hybridizations are realized simultaneously with two probes, complementary to the 5' D4Z4 region and to a 200Kb region of the 4q35.2 (located 3.5 Mb upstream from D4Z4) respectively. Since FSHD is a progressive pathology, we have chosen to focus on three different cell types fetal and adult primary myoblasts and fibroblasts from patients or controls, as well as induced pluripotent stem cells (IPSc),derived from primary fibroblasts. In a second step, the distribution of the D4Z4 locus will be followed during differentiation into several lineages (teratomas, embryoïd bodies, differentiated cells). Our results show that both regions are localized at the nuclear periphery, and on that hybridization volumes of signals display a significant difference between patients and controls. Therefore, the degree of chromatin relaxed could correlate to the pathology. Moreover, signals are more colocalized in patient cells, suggesting an interaction between both sequences, which could undergo the same regulatory mechanisms. Signals distribution and colocalisation tend to demonstrate a fetal origin of FSHD pathogenic processes. Hence, we will consolidate our data by using iPSCs as a model of the pathology, from the pluripotent to the differentiated state. These approaches should bring further insights into the underlying mechanisms of FSHD in order to identify target genes and to understand by which mechanism, a decrease in the number of a repetitive macrosatellite sequence, D4Z4, leads to this muscular dystrophy.

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DYNAMIC LINK OF 5-HYDROXYMETHYLCYTOSINE WITH DNA REPLICATION DURING EARLY S-PHASE IN MOUSE EMBRYONIC STEM CELLS AND SOMATIC CELLS

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Epigenetic changes, such as the methylation of DNA cytosine (resulting in 5mC) and histone modification, regulate gene expression in cells and development in mammals. 5mC is demethylated throughout the genome in early-preimplantation mouse embryos and in primordial germ cells migrating into the genital ridge. Recently, a new demethylation mechanism has been discovered by which the hydroxylation enzymes belonging to the Tet family can convert 5mC into 5-hydroxymethylcytosine (5hmC). Embryonic demethylation is initiated by Tet3 in mouse zygotes as an active event but is subsequently accomplished in a cell-cycle-dependent manner. In this study, we found that 5hmC was accumulated in the chromosomal regions replicating during early S in mouse embryonic stem cells (ESC) and human iPS cell-derived differentiated cells. A dynamic link between global gene activity and replication timing has been suggested, whereby chromosomal bands replicating in early S are usually active while those portions replicating later are inactive. Mouse Y chromosome and human inactive X chromosome replicating in late S were entirely negative for 5hmC, excluding the active pseudo-homologous region of human X chromosome. The fact that mutant ESC lacking DNA methylation activity possesses the self-renewing potency suggests that 5mC and 5hmC do not affect replication timing nor higher order chromatin structure at the chromosomal level in ESC. A recent study has demonstrated that PGC7/Dppa3 protects maternal pronuclei from the Tet3 enzymatic activity in zygotes, but is hardly detected in somatic cells. Thus, our findings suggest that alternative chromosome-binding factors might exclude the Tet activity from inactive regions to protect 5mC from reprogramming activity, leading to the selective recruitment of Tet onto active chromosomal regions where it can convert unfavorable 5mC.

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HP1Γ AFFECTS THE DIFFERENTIATION CAPACITY IN P19 EMBRYONAL CARCINOMA CELLS.

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Heterochromatin protein 1 (HP1) is an epigenetic modulator that binds to the methylated lysine 9 residue of histone H3 (H3K9me), which is a hallmark histone modification for transcriptionally silenced heterochromatin. In vertebrates, three isoforms of HP1 exists as follows: α, β, and γ. These isoforms share highly conserved domains and are associated with H3K9me, which are important for gene silencing. In general, HP1α and HP1β are found at constitutive heterochromatin, while HP1γ is distributed in both heterochromatic and euchromatic regions. Consistent to this notion, HP1γ, but not HP1α and HP1β, was found to associate with actively transcribed gene regions and plays a role in transcriptional activation or in transcriptional elongation. Here, we have studied the role of HP1γ in differentiation capacity of embryonal carcinoma (EC) cells. EC cells have numerous morphological and biochemical properties in common with pluripotent stem cells such as embryonic stem (ES) cells. In particular, P19 EC cells differentiate into cardiomyocytes in the presence of dimethyl suloxiside (DMSO), and commit to neural lineage directly by an addition of retinoic acid (RA). However, P19 cells basically keep their undifferentiated state and show no sign of differentiation even after embryoid body (EB) formation in normal culture condition. Therefore, to examine whether HP1γ affects the differentiation potential of P19 cells, we have established HP1γ over-expressing P19 cell lines (HP1γ-P19). Like parental P19 cells, slowly proliferative HP1γ-P19 cells continued to express pluripotent maker genes and were positive for alkaline phosphatase in undifferentiated state. The analysis by flow cytometry (FCM) revealed that both HP1γ-P19 cells and wild type cells were found to express similar levels of E-Cadherin and that neither showed any expression of Nanog. These results suggested that the stem cell state of P19 cells with the property of epiblasts was not affected by the expression of HP1γ. In contrast, HP1γ-P19 cells showed spontaneous morphological differentiation after EB formation without any treatment of chemical inducer. RT-PCR analysis revealed that EB cells of HP1γ-P19 expressed various differentiation maker genes such as Brachyury, Flk-1, Nkx2.5, Gata4, AFP and Nestin. In addition, HP1γ-P19 cells differentiated into Tuji-1 positive neuro-ectodermal cells and endoderm-like cells expressing the transcription factors Gata6 and Sox17 with a characteristic morphology. Furthermore, HP1γ-P19 cells differentiated into cardiac cells with spontaneous beating efficiently, which was confirmed by immuno-staining with tropomyosin C and cardiac troponin T. Finally, HP1γ-P19 cells developed teratoma with spontaneously differentiated tissues such as cerebrum. Thus HP1γ- P19 cells could give rise to all three germ layer cells in EB without any drug treatment. Therefore HP1γ affect the autonomous differentiation potential in P19 cells, and could play major roles on the decision of cell fates in pluripotent stem cells.

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FUNCTIONAL DISSECTION OF HISTONE H3 LYSINE 27 DEMETHYLATION IN NEURAL DIFFERENTIATION AND CORTICAL DEVELOPMENT

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Polycomb-mediated trimethylation of lysine 27 on histone H3 (H3K-27me3) has emerged as a bona fide epigenetic repressive mark that can be maintained through cell division. Three lines of evidence have revealed however the unexpected dynamics of this mark: i) the comparison of genome-wide chromatin modifications between mouse embryonic stem cells (ESC) and neural precursors (NP), that revealed that Polycomb target genes are kept in a poised state by a bivalent chromatin signature that features both H3K4me3 and H3K27me3 marks and that in the ESC to NP transition gene activation correlates selectively with losses in H3K27me3; ii) the description of unanticipated genome-wide changes in H3K27me3 that accompany the entire process of neural fate acquisition rather than simply the exit from the pluripotent state, making the developing central nervous system (CNS) a paradigm-setting model to interrogate the function of H3K27me3 in neural stem cell (NSC) dynamics and neuronal differentiation (Testa, Bioessays 2011); and iii) our identification of Jmjd3 as a H3K27 demethylase that is required for ESC neural commitment through the direct activation of key drivers of neurogenesis (De Santa et al. Cell 2007; Burgold et al. PLoS One 2008). Yet, despite evidence of its involvement in early neural commitment, the role of Jmjd3 in the emergence and maturation of the mammalian CNS *in vivo* remained unknown. Here we describe the first functional dissection of the role of Jmjd3 and H3K27 demethylation in the mammalian CNS. Inactivation of Jmjd3 in the mouse led to perinatal lethality, and our characterization of this phenotype uncovered a key role for Jmjd3 in the maturation of embryonic neuronal networks along with specific defects in the balance of self-renewal and differentiation in the NSC compartment. Specifically, we report the effects of perturbing Jmjd3 function during corticogenesis through a combination of *in vivo* and in vitro approaches and a unique panel of murine strains that enable the timed ablation or overexpression of this enzyme. With these tools we relate the *in vivo* modulation of Jmjd3 in radial glial cells to its regulation of proliferation and differentiation using two well-defined in vitro differentiation systems: i) the differentiation of E13.5 NSC that defined a lineage hierarchy whereby tripotent neural progenitors give rise to neurons, astrocytes and oligodendrocytes through a stereotypical sequence of intermediate bipotent progenitors that is reset at each passage in culture; and ii) the differentiation of ESC into a homogenous population of cortical glutamatergic neurons through the intermediate generation of homogeneous Pax6-positive, radial glia-like precursors. Our findings show that Jmjd3 regulates the transition between self-renewal and differentiation selectively in apical progenitors, with its loss resulting in an expansion of the radial glia compartment and an impairment in neuronal differentiation. We then combine time-lapse videomicroscopy with global analysis of gene expression and chromatin (through RNA-seq and ChIP-seq), to uncover the mechanistic basis underlying Jmjd3 control of neural fate, including the identification of specific target genes during corticogenesis. Finally, we integrate experimental data with reverse engineering inference algorithms to validate the gene networks that are orchestrated by Jmjd3 in the neural stem cell compartment.

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HIPPOCAMPAL EPIGENETIC MODIFICATION AT THE BRAIN-DERIVED NEUROTROPHIC FACTOR GENE INDUCED BY AN ENRICHED ENVIRONMENT.

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Environmental enrichment is an experimental paradigm that increases brain-derived neurotrophic factor (BDNF) gene expression accompanied by neurogenesis in the hippocampus of rodents. In the present study, we investigated whether an enriched environment could cause epigenetic modification at the BDNF gene in the hippocampus of mice. Exposure to an enriched environment for 3-4 weeks caused a dramatic increase in the mRNA expression of BDNF, but not platelet-derived growth factor A (PDGF-A), PDGF-B, vascular endothelial growth factor (VEGF), nerve growth factor (NGF), epidermal growth factor (EGF), or glial fibrillary acidic protein (GFAP), in the hippocampus of mice. Under these conditions, exposure to an enriched environment induced a significant increase in histone H3 lysine 4 (H3K4) trimethylation at the BDNF P3 and P6 promoters, in contrast to significant decreases in histone H3 lysine 9 (H3K9) trimethylation at the BDNF P4 promoter and histone H3 lysine 27 (H3K27) trimethylation at the BDNF P3 and P4 promoters without any changes in the expression of their associated histone methylases and demethylases in the hippocampus. The expression levels of several microRNAs in the hippocampus were not changed by an enriched environment. These results suggest that an enriched environment increases BDNF mRNA expression via sustained epigenetic modification in the mouse hippocampus.

Poster Board Number: F-2198

GLOBAL CHROMATIN DYNAMICS DURING HEMATOPOIETIC STEM CELL DIFFERENTIATION

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Epigenetic mechanisms evidently play a role in determining the identity of stem cells, but how they precisely contribute is partially unknown. A proposed hallmark of embryonic stem cell (ESC) pluripotency is a globally open and dynamic chromatin structure, which become condensed and less accessible upon differentiation into mature cells. However, whether global chromatin differences serve as a shared mechanism to influence lineage potential in adult stem cells remains to be elucidated. We used hematopoietic stem cells (HSCs) and their progeny to determine the differences in global chromatin conformation that accompany differentiation into mature blood cells. General DNaseI sensitivity assays revealed that HSCs and myeloid progenitors have a significant increase in condensed chromatin compared to ESC, but a much more nuclease-sensitive chromatin structure than fully differentiated cells. Surprisingly, the changes in global chromatin accessibility between these cell populations did not correlate with significant changes in global levels of active and repressive histone modifications, or with global levels of DNA methylation. However, immunofluorescence image analysis for the H3K9me3 histone mark revealed that hematopoietic stem and progenitor cells show distinct heterochromatin conformation and nuclear architecture compared to mature cells. Additionally, high-resolution 3-dimensional images of HSCs and differentiated cells have been acquired using soft X-ray microscopy, enabling us to precisely quantify the levels of condensed and open chromatin, and interrogate differential chromatin organization. Overall, our results demonstrate that HSCs have a more open chromatin structure compared to mature hematopoietic cells, correlating with their higher developmental potential. Elucidating the underlying chromatin structure of HSCs and its dynamics during differentiation is essential to comprehend the role of chromatin remodeling and epigenetics in the transition between these stages. In addition, comparison to leukemic stem cells will help us understand how chromatin is dysregulated in leukemogenesis.

Poster Board Number: F-2200

NOVEL TRANSCRIPTIONAL COACTIVATOR COMPLEX IS REQUIRED FOR CELLULAR REPROGRAMMING

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The establishment and maintenance of ES cell-specific transcription is known to be regulated by a variety of sequence-specific transcriptional activators such as Oct4, Sox2 and Nanog. These ES cell core transcription factors can potentiate their own gene expression and activate expression of each other, which forms auto-regulatory circuits as well as feed-forward loops. Despite the well-documented functions of these core activators, very little is known about an ES cell specific co-activator(s) involved in ES cell transcriptional core circuits. Here we identified two multi-subunit stem cell co-activator complexes (SCC-A and SCC-B) by in vitro biochemical purification. Surprisingly, SCC-B complex is identical as the Xeroderma pigmentosum group C (Xpc)-Rad23b-Centrin2 (Cetn2) nucleotide excision repair (NER) complex and this complex is important to support full activation of Nanog expression as well as other ES cell specific genes *in vivo*. We sought to test the role of the SCC-B complex during cellular reprogramming by knocking down each component. Intriguingly, loss of Xpc or Rad23b led to a drastic reduction of iPS cell formation. On the other hand, knockdown of Cetn2 had minor effects on iPS cell derivation, which is consistent with our in vitro transcription assay showing Cetn2 is not essential for the co-activator activity. Rad23a and Rad23b are functionally redundant in regards to NER activity as either Rad23a or Rad23b depleted cells are proficient in NER. To our surprise, Rad23a depleted cells had no obvious defect on iPS cell derivation. These results imply that Rad23b has essential functions during iPS formation beyond NER activity, which might be its transcriptional role as part of the Xpc/Rad23b/Cent2 complex. In summary, the novel ES cell co-activator complex, SCC-B/Xpc complex, is required for reacquisition of pluripotency during cellular reprogramming.

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Mesenchymal Cell Lineage Analysis

Poster Board Number: F-2201

NOVEL HUMAN BONE MARROW DERIVED MESENCHYMAL STROMAL CELLS LACKING ADIPOGENIC DIFFERENTIATION POTENTIAL SUPPORT PRIMITIVE HUMAN CD34-NEGATIVE HEMATOPOIETIC STEM CELLS

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(Background) We recently identified human cord blood-derived CD34-negative (CD34-) SCID-repopulating cells (SRCs) using intra-bone marrow injection (IBMI) technic (Blood 101:2924, 2003). These CD34- SRCs provide a new concept for the hierarchy in the human hematopoietic stem cell (HSC) compartment. Furthermore, we succeeded to highly purify these CD34- SRCs using 18 lineage specific antibodies (Exp Hematol 39:203, 2011). On the other hands, HSC niche is thought to exist in the bone marrow (BM). In the mouse study, it has been reported that osteoblasts, vascular endothelial cells, CXCL12-abundant reticular cells and nestin-positive mesenchymal stem/stromal cells (MSCs) played an important role to organize murine HSC niches. However, it is not clearly understood which cells play a pivotal role in the maintenance of self-renewal capacity and dormancy of primitive HSCs in the human BM niche. (Objectives) The aim of this study was to prospectively isolate/identify human BM niche cells and investigate their functional characteristics. (Results) First, human BM-derived Lin-CD45- cells were subdivided into 4 fractions according to their expression levels of CD271 and SSEA-4 by FACS. In the results, we succeeded to isolate three types of MSCs from CD271+SSEA-4- (CD271 SP), CD271-SSEA-4- (DN) and CD271+SSEA-4+ (DP) fractions. All of these three MSCs expressed MSC surface markers including CD29, CD44, CD73, CD90 and CD105. In addition, two of these MSCs, isolated from CD271 SP and DN fractions, could differentiate into osteoblasts, adipocytes, and chondrocytes. Interestingly, the MSC isolated from DP fractions (DP MSC) could also differentiate into osteoblasts and adipocytes, but could not differentiate into adipocytes. Then, we assessed CD34- SRC-supportive activity of these three MSCs. First, the 18Lin-CD34- cells were cocultured with three MSCs for 1 week, respectively. Next, the cells were collected and transplanted into NOD/SCID mice by IBMI to investigate SRC activity. In the results, the highest human CD45+ cell engraftment was observed in the mouse BM received 18Lin-CD34- cells cocultured with DP MSC 8 weeks after transplantation. The DP MSCs expressed higher level of several HSC-supportive genes and nestin gene than other MSCs. In addition, we observed Lin-CD34+CD38-CD90+CD45RA- cells were generated from 18Lin-CD34- cells during cocultures with DP MSCs. It is recently reported (Cell Stem Cell 1:635, 2007, Science 333:218, 2011) that these Lin-CD34+CD38-CD45RA-CD90+ cells contained most primitive human CD34+CD38- SRCs. In order to evaluate the SRC activity of these Lin-CD34+CD38-CD90+CD45RA- cells generated from 18Lin-CD34- cells in vitro, the Lin-CD34+CD38-CD90+/-CD45RA- cells were sorted by FACS and then transplanted into NOD/SCID mice by IBMI. Twelve weeks after transplantation, 8 out of the 16 mice that received Lin-CD34+CD38-CD90+CD45RA- cells (400 to 3500 cells/mouse) were repopulated with human cells with multi-lineage reconstitution. In contrast, none of the 16 mice that received Lin-CD34+CD38-CD90-CD45RA- cells (1500 to 7000 cells/mouse) were repopulated with human cells. (Conclusion) These findings elucidate that human DP MSCs can support very primitive human CB-derived CD34- SRCs in vitro. Therefore, application of DP MSC might be useful for *ex vivo* HSC expansion. In addition, these CD34- SRCs seem to be more immature than CD34+CD38- SRCs which have been thought to be most primitive human HSCs.

Poster Board Number: F-2202

CHARACTERIZATION OF PERIDUCTAL AND PERIALVEOLAR FIBROBLASTS IN NORMAL HUMAN BREAST TISSUE

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The human breast is characterized by the presence of somatic stem/progenitor cells. Normal breast stem cells are dependent on a relevant *in vivo* microenvironment, i.e. "humanized stroma" for optimal growth and morphogenesis. Morphogenesis includes duct- as well as lobule formation, and we have previously found evidence for a breast stem cell hierarchy with a strong topographical component. Here we compared the ductal and lobular stroma and found that they were CD13+/CD26neg and CD13low/CD26+, respectively. These fibroblast subpopulations were first analyzed by FACS of freshly isolated normal breast organoids incubated with Aldefluor, EpCAM, CD13 and CD26, and subsequently sorted into Aldefluor+/CD13high/CD26low and CD13low/CD26high stromal subpopulations, respectively. By immunocytochemistry four other markers previously implicated as mesenchymal stem cell markers, NG2/neuron-glial antigen 2, CD73/ecto-5′ -nucleotidase, CD90/Thy-1 and CD105/endoglin, were used to distinguish the two populations. Staining for CD31 excluded an endothelial origin. Higher expressions of NG2 and CD105 and lower expressions of CD73 and CD90 was found in the Aldefluor+/CD13high/CD26low fibroblast subpopulation as compared to the CD13low/CD26high fibroblast subpopulation. Once separated there was only little evidence of phenotypic interconversion between the two cell populations under identical culture conditions for eight passages. Based on these observations we propose that lineages exist also within interstitial stroma and that these correlate spatially with distinct parenchyma within the human breast. This opens for the possibility to examine the role of stromal compartmentalization for normal breast morphogenesis and cancer development.

Poster Board Number: F-2203

COMPARISON OF INTRA-ARTERIAL AND INTRA-VENOUS TRANSPLANTATION OF HUMAN BONE MARROW MESENCHYMAL STEM CELLS SUPPRESSED ISCHEMIC NEURONAL CELL DEATH.

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A series of observations in rodent and primate models have suggested that a potential therapy for ischemia in the central nervous system is the administration of adult stem/progenitor cells from bone marrow referred to as mesenchymal stem cells or multipotent mesenchymal stromal cells (MSCs). However, the underlying

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mechanisms of MSCs' neuroprotection have not been established in detail. Some of reports suggest that intra-venous transplantation of hMSCs suppresses diseases in rodent and clinical trials, and mixed-cluture studies of various cells and hMSCs with transwell chamber are influenced by hMSCs. Therefore, it is suggested that a part of function on hMSCs is mediated by humoral factors. However, we still want to search appropriate route to transplant hMSCs. In the present study, we transplanted hMSCs into mouse after transient focal ischemia with intra-venous (iv) or intra-artery (ia), and compared the effect of hMSCs on the ischemic neuronal cell death. Male C57/BL6 mice were subjected to 1 hour transient middle cerebral artery occlusion (tMCAO) by a suture method. Immediately after reperfusion, the animals were transplanted hMSCs at 10^6 to 10^4 cells into either right jugular vein (iv) or left common carotid artery (ia). Control animals were given HBSS as a vehicle in same way. During the experiment, the animals were also evaluated neurological deficit by modified neurological severity score (mNSS) and the animals which showed 5 or more of mNSS 1 hour after ischemia were transplanted cells or vehicle. Two days after reperfusion, the infarct volume was evaluated with triphenyl tetrazolium chloride (TTC) staining. The cell delivery was demonstrated by PKH26-labeled hMSCs and EGFP-hMSCs transplantation. Infarct volume of control animals between ia and iv showed no difference. The infarct volume transplanted hMSCs (ia, 10^6) was significantly suppressed to 43% of control animals ($p<0.01$) and decreased the effect in the cell number-dependent fashion. The infarct volume transplanted hMSCs (iv, 10^6) tended to be suppressed, but no significant differences as compared with control one. One hour after ischemia, control animals showed 6.63 mNSS and no significant differences were observed in the animals treated any number of hMSCs (ia) transplant group. The mNSS were significantly improved 10^6 and 10^5 hMSCs (ia) animals. Demonstrating the cell delivery in the brain, huge number of the hMSCs were observed in the entire of brain after transplantion hMSCs (10^6) with intra-arterial although intra-venous transplantation of hMSCs (10^6) was observed only a few cells. The intra-arterial transplantation of hMSCs (10^6) were observed the cells in the brain 2 days after transplantation. These results suggest that greater number of hMSCs delivery with intra-artery transplantation might be better way to suppress neuronal cell death after ischemia. Further study requires how and what mechanism hMSCs play protective role mediated by cell-cell contact or not.

Poster Board Number: F-2204

WEEK BY WEEK CHANGES IN THE TRANSCRIPTIONAL ONTOGENY OF HUMAN FIRST TRIMESTER FETAL AND PLACENTAL MESENCHYMAL STEM CELLS

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Although MSC can be isolated from fetal and adnexal tissues across gestation, early fetal MSC display more primitive characteristics than their later gestation counterparts. Placental/membranous tissues are increasingly suggested as therapeutic alternatives to cells derived directly from the fetus. To date microarray studies comparing gene expression profiles between MSC from different origins, have paid little attention to the effect of gestational age (GA). Given the huge ontological changes during a time when the fetus increases 4-5 fold in length between 8-12 weeks, we hypothesised that earlier MSC have a higher degree of primitiveness than later 1st trimester pregnancy related stem cells. Using Illumina beadchip microarray technology, we characterized the developmental transcriptome of first trimester MSC from fetal bone marrow (BM) compared to that from adnexal structures across multiple time points (8wks-12wks). Within the 1st trimester, we found 2624 genes differentially expressed in earlier (8/9 weeks GA) compared to later BM-MSC (10-12 weeks GA), with a strong transition point at 10 weeks gestation. Before this, a strong signature around the pluripotency network was observed in early BM-MSC with an over presentation of *OCT-4* target genes. This was then downregulated after 10 weeks, when differentiation and immune response genes were upregulated. Additionally, a shift in gene ontogeny was also observed between early and late BM-MSC. Pathways involved in cellular assembly including nucleic acid metabolism, DNA replication and embryonic development were over represented in early BM-MSC whereas pathways over represented in late BM-MSC were dominated by organ developmental and function. Interestingly, no transition was observed in placental MSC, reflecting their less complex ontogeny in comparison to those from the fetus, in which the switch from embryo to fetus occurs at around this 10 week time point. This study documents profound transitional changes along a pluripotency to differentiation gradient in first trimester fetal but not adnexal MSC, which may have implications for translational cell selection.

Poster Board Number: F-2205

CHARACTERIZATION AND EXPRESSION OF PLURIPOTENCY MARKERS IN HUMAN DENTAL PULP STEM CELLS

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The tooth is composed of hard and soft tissues that are derived from the oral epithelium and the dental mesenchyme. In the present study, we characterized and examined the expression of pluripotency markers, such as Oct-3/4, Sox-2 and Nanog both at mRNA and protein levels in human dental pulp stem cells (DPSCs). DPSCs were harvested from the pulp tissue after impacted third molars extractions in orthodontic treatment patients (approximately 18 yrs old). Isolated DPSCs were analyzed for the expression of cell surface and intracellular markers by flow cytometry. The ability of DPSCs for osteogenesis and adipogenesis was examined by following previously published protocols. The differentiated cells were assessed by cytochemical staining and the expression of lineage specific markers by RT-PCR. Further, the expression of pluripotent stem cell transcription factors was evaluated by RT-PCR, immunofluorescence and western blotting analysis. Isolated DPSCs showed a typical fibroblastic morphology with an ability to form colony forming units (CFU) following their culture in a serum supplemented ADMEM. DPSCs positively expressed the cell surface markers, such as CD29, CD44, CD73, CD90, CD105 and an intracellular marker, vimentin, but not the hematopoietic cell lineage markers, such as CD 34 and CD45. Upon induction in lineage specific media, DPSCs were differentiated into osteocytes and adipocytes as confirmed by the deposition of calcium matrix (Alizarin red and von Kossa staining) and the accumulation of lipid droplets (Oil red O staining), respectively. This was further evidenced by the expression of genes involved in osteogenesis [osteonectin (ON) and runt-related transcription factor 2, (Runx 2)] and adipogenesis [(adipocyte fatty

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acid binding protein 2 (aP2) and peroxisome proliferator-activated receptors γ2(PPAR- γ2)]. These biological properties of DPSCs were similar to mesenchymal stem cells (MSCs). In addition, RT-PCR and western blot analysis showed the expression of pluripotency markers, such as Oct-3/4, Sox-2 and Nanog both at mRNA and protein levels in DPSCs. Thus, these factors may have a potential role in influencing the high proliferation and broader differentiation ability of DPSCs. In conclusion, the results of this study demonstrate that, DPSCs represent an ideal alternative autologous cell source possessing the properties of enhanced proliferative potential and capacity to multi lineage differentiation, and could become feasible tools for dental tissue engineering. This study was supported by grants from BioGreen21, Rural Development Administration (Grant No. 20110701-305-533-001-02-00), Republic of Korea.

Poster Board Number: F-2206

HUMAN CHORIONIC PLATE CONTAINS MSC-LIKE POPULATION WITH A COMPARATIVELY RAPID PROLIFERATION RATE THAN MARROW MSCS

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Background: Extensive proliferation capacity as well as multilineage differentiation potential of mesenchymal stem cells (MSCs) render them as promising cellular materials for application in cell-based threatment of tissue defects in particular as bone and cartilage extensive damages. To promote regeneration in tissue defects, however, enormous cell number is required. Considering the scarce number of MSCs from marrow tissue, *in vitro* propagation of the cells becomes inevitable. In routine cell culture lab at least 3-4 weeks would take to multiply the cells into sufficient number. In the search of alternative source having MSCs with rapid rate of proliferation, we found human chorionic plate as an appropriate source of fetal MSCs. In this study we compared chorionic plate-derived MSCs with bone marrow-derived MSCs. Methods: chorionic plate was collected from the hospital and subjected to the enzymatic digestion using collagenase IV. Released cells were then plated and culture-expanded through several successive passages. Passaged-3 cells were characterized in terms of surface antigenic phenotype as well as multilineage differentiation potential. The isolated stem cells appeared to be rapidly proliferative. Therefore population doubling time (PDT) as well as colonogenic activity, as two important indicators of stem cell proliferation rate, were calculated for the cells and compared to those of human marrow MSCs. Results: At primary culture of chorionic plate cells, there were some fibroblastic colonies along with some round as well as star-shaped cells. Fibroblastic cells gradually dominated the culture. The majority of these cells were positive for mesenchymal markers including CD105, CD44, CD90, and CD73. Hematopoietic cell lineage markers such as CD11b, CD34 and CD45 were expressed on small percentages of the cells. Regarding differentiation capacity, chorionic plate-derived stem cells, similar to their marrow counterpart, succeeded to give rise to bone, cartilage and adipose cells. Adipogenic as well as chondrogenic differentiation potential of the chorionic stem cells, however, seemed to be weak compared to that of marrow MSCs. The interesting point was the significant difference in the proliferation rate of two cells. The PDT for the chorionic cells was determined to be 27.62±2 while this value tended to be 49.57±0.8 for the marrow MSCs. Furthermore the chorionic stem cells produced more colonies compared to those from marrow tissue. Conclusion: this study describes a MSC-like population of rapidly- proliferative cells from human chorionic plate which is a waste component of fetal tissues. Such cells would be of interest since using these cells for regenerative purpose can shorten the time of construct preparation for cell-based treatment of tissue defects.

Poster Board Number: F-2207

CCL5 SIGNALING REGULATES MULTIPOTENCY OF HUMAN ADIPOSE TISSUE DERIVED STEM-LIKE CELLS

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Adult mesenchymal stem-like cells (MSCs) differentiate under appropriate conditions into various mesodermal derivatives. It has been suggested that similarly to embryonic stem cells, stemness of MSCs is supported by the regulatory networks containing the key transcription factors (OCT4, NANOG, SOX2 and KLF4) in their core. These factors are associated with pluri- and multipotency and form the basis of regulation of stem cell renewal and differentiation. One of the potential clinical applications of isolated and cultured MSCs relies on their capacity for targeted and controlled migration and engraftment to sites of injury, inflammation and tumors. Furthermore, it is believed that under inflammatory conditions, endogenous MSCs are activated and induced for directional movement to sites of injury where they participate in tissue regeneration processes. Signals of inflammation are partly mediated by chemokines. Chemokines are secreted signalling molecules regulating numerous functions of cells such as proliferation, establishment of cellular polarity, migration, angiogenic capacity and differentiation. Chemokines stimulate the target cells through binding to their receptors belonging to G-protein-coupled receptor family. Chemokine receptors have been described in bone marrow derived MSCs. However, chemokine signalling in molecular terms has not been yet investigated in adipose tissue derived stromal cells (AdMSTCs) containing a subset of MSCs. In the current study, we assumed that human adipose tissue derived MSCs express functional chemokine receptors, and responsiveness to chemokine signalling is one of the attributes of biologically active MSCs. We show that repertoire of chemokine receptors expressed in AdMSTCs includes CCR1, CCR3 and CCR5, representing a group of receptors able to interact with CCL5 or RANTES. CCR1, CCR3 and CCR5 known to form a gene cluster suggesting that they may be expressed in the same cells. A portion of CCR1 positive cells varies within populations of AdMSTCs from 4% up to 25%. Moreover, the level of CCR1 expression positively correlates with expression level of the multipotency factors OCT4, NANOG and SOX2. We show that CCL5 receptors are biologically active. Stimulation of AdMSTCs with CCL5 leads to activation of ERK and AKT kinases and NFκB signalling pathway. As a result, expression of NFκB target genes such as IL-6, MMP1 and MMP9 is elevated within 8 h. Besides, in response to CCL5, expression of anti-apoptotic gene SURVIVINE increases, whereas pro-apoptotic genes BID and BAXa are down-regulated. These data suggest that CCL5 may be a positive regulator of AdMSC proliferation inducing as well anti-apoptotic effects. Finally, we show that OCT4, SOX2 and NANOG expression levels as well as expression of CCR1, CCR3 and CCR5 increase in response to CCL5 stimulation within 48h. As a result, differentiation capacity of AdMSTCs increases. Comparing with their untreated counterparts, the CCL5-treated cells exhibit elevated response to several growth factors, e.g. NGF and BMP2, inducers of neuronal and osteogenic differentiation, respectively. Taken together, our data suggest that MSCs possess functional receptors for CCL5 that should be assessed as surface antigens for MSC immunopurification.

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Poster Board Number: F-2208

IDENTIFICATION AND ENRICHMENT OF AN ESC LIKE CLONOGENIC MESENCHYMAL STEM CELLS IN HUMAN PLACENTA (AM-CMSC) FOR REGENERATIVE MEDICINE

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Neonatal placenta is a tissue supporting the neonatal development from embryo and fetus that should consists with various superior functioning embryonic and fetal stromal stem/progenitor cells with greater proliferation ability and the immune safety. By a systemic screening of mesenchymal stromal stem/progenitor cells isolated from the placenta tissue, we have identified a subset of clonogenic mesenchymal stem cells (AM-cMSC) from the placenta amnion membrane stromal layer (Am-MSCs), by a CD34 cell antigen FACS sorting. We found the isolated AM-cMSCs exhibited sphere-like clonogenicty in early passages and expresses pluripotent embryogenic stem cell (ESCs) like characteristics. The isolated AM-cMSC subpopulation can be expanded, while maintaining the stemness genes and the phenotype marker expressions in culture over a month. These cells simultaneously expressed embryonic (SSEA membrane sphincolipids, Oct-4, Nanog, Rex-1, Sox-2), stemness (e.g. CD117, CD34, CD133, CD146) surface antigens, in addition to present various lineage markers including: mesenchymal (CD29, CD90, CD73, CD105, CD106), hem-angiogenic (e.g. AC133, CD34), myo-nurogenic (e.g. CD54, nestin, NSE). AM-cMSCs exhibits excellent trans-dermal differentiation potentials in vitro, at least including adipogenesis, osteogenesis, chondrogenesis, neurogenesis, vasculogenesis, hepatogenic & myogenesis, tested to date. Current study provides a better insight into the ESCs like clonogenic stem cells can be derived from non-embryonic neonatal placental tissue. The proliferative and differentiation potentials found from this study indicates that a great potential of AM-cMSCs to be used for clinical regenerative therapies. More *in vivo* animal studies as well the in vitro molecular profiling characterization in gene expression and protein synthesis are under going. Acknowledgement: The study is supported by an internal collaboration project among the Graduate Institute of Medical Sciences, Taipei Medical University, and OBGYN research Center, Cathy General Hospital, and Genomic Research Center, Academia Sinica. A partly support financially of this study is obtained from Sino Health Bank, Taiwan.

Poster Board Number: F-2209

TOWARDS IDENTIFICATION OF SPECIFIC HUMAN ADULT MESENCHYMAL STEM CELL MARKERS

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Mesenchymal stem cells have been identified in a wide variety of tissues and have properties that make them amenable to therapeutic use. However, for mesenchymal stem cells to be accepted in clinical practice, techniques for their isolation and purification need to be standardized. To date, there is no conclusive marker(s) for the specific isolation of mesenchymal stem cells. Our aim was to identify specific mesenchymal stem cell markers by comparing the phenotype of cells from tissues that are rich and deplete of mesenchymal stem cells. Initially, we assessed the ability of, bone marrow, breast adipose, foreskin fibroblasts and olfactory tissue cells to demonstrate mesenchymal stem cell properties. By examining their tri-lineage differentiation potential (adipocytes, osteocytes and chondrocytes), inhibition of T cell proliferation and colony formation, it was apparent that bone marrow and breast adipose cultures are rich in mesenchymal stem cells while olfactory tissue and foreskin fibroblast cultures are relatively deplete of mesenchymal stem cells. Subsequently, the phenotype of tissue cells was thoroughly assessed using immuno-fluorescence, flow-cytometry, proteomics and antibody arrays. Phenotypic analysis revealed that common mesenchymal stem cell markers and fibroblast associated markers fail to discriminate between cultures rich and deplete of mesenchymal stem cells and that all tissue cells tested had remarkably similar phenotypes. However, by meticulous examination and comparison of the phenotype of tissue cultures rich and deplete of mesenchymal stem cells, we were able to identify markers indicative of mesenchymal stem cell

Poster Board Number: F-2210

CELLS ISOLATED FROM HUMAN ARTICULAR CARTILAGE EXPRESS STEM CELL MARKERS SSEA3, SSEA4, CD34, FRIZZLED 9, ABC TRANSPORTER ABCG2, ALDEHYDE DEHYDROGENASE 1

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Background: The view of articular cartilage as a non-regenerating organ has been challenged in recent years. Several studies attempted to identify progenitors in cartilage by the use of different combinations of typical mesenchymal stem cell (MSC) markers. We analyzed ABCG2 expression, responsible for formation of Side Population, and some other proteins, recently characterized as markers for embryonic and other stem cells. Methods: Cells were isolated from human articular cartilage and cultivated in monolayer. Expression of cell surface markers was measured by flow cytometry. Aldehyde dehydrogenase 1 (ALDH) activity was analyzed using fluorescent substrate Aldefluor by flow cytometry. Gene expression of ABCG2 and some other markers was identified by QPCR with TaqMan probes. Results and Discussion: Surface markers Notch1, CD146 (MCAM), CXCR6 and some typical MSC markers CD44, CD90, CD166 were expressed poorly few days after isolation, but the expression dramatically increased during cultivation in monolayer. Expression of SSEA4 and c-Kit only slightly increased, but never exceeded 20% of cells. Another group of analyzed markers, usually attributed to stem/progenitor cells CD34, NCAM, CXCR4, Frizzled9 and SSEA3, demonstrated different pattern of expression - they were identified at their maximal level few days after isolation (5-15% for CD34, up to 30-50% for the others) and later their expression decreased. Possibly that monolayer culture was not suitable for these cells, what was shown for myogenic progenitors from muscle. ABCG2 expression was found only for 1-3 days after isolation, reaching up to 30% of cells, while after few days of cultivation almost no expression was found. Immunohistochemical staining of cartilage identified these cells in the region of merge of synovial membrane, cartilage and subchondral bone. ALDH activity was also strongest in freshly isolated cells (up to 40%) and almost disappeared in 8 weeks of cultivation in monolayer. QPCR analysis revealed that ABCG2 gene was expressed in isolated cells about 1000 times less than housekeeping gene. Nanog was expressed at the same level, OCT4 - about 10 times more and Sox2 - about 10 times less. Conclu-

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sion: We demonstrated for the first time the presence of stem cell markers SSEA3, SSEA4, Frizzled 9 and ALDH activity in cells isolated from human articular cartilage and their expression during cultivation in monolayer. We also showed the localization of ABCG2 positive cells in the tissue. As it was shown recently that SSEA3+ cells from different tissues were multipotent and could differentiate into cells from all 3 germ layers, and only SSEA3+ cells could generate iPS, this information could be important for identification of stem/progenitor zone in cartilage and useful for tissue engineering.

Poster Board Number: F-2211

MESENCHYMAL PRECURSOR CELLS IN THE MOUSE AND HUMAN PROSTATE

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Most men aged 50 or over experience symptoms of benign prostatic hyperplasia (BPH), including nocturia, poor stream and frequency. Histologically, BPH is associated in most cases with hyperplasia of mesenchymal cells. Aberrant growth of mesenchymal progenitor cells has been proposed as an etiology of BPH. The aim of this study was to identify markers of mesenchymal precursor cells in the prostate using cell sorting (magnetic, MACS and fluorescent, FACS). Imuunohistochemistry data have shown that CD90 and CD105 positive cells were located in the stroma but not in the epithelium of mouse prostate. FACS analysis showed that the CD90+ cells are a subset of the CD105+ cells. Immunocytochemistry showed expression of putative stem cell markers Sca-1, Trop2 and BMI-1 in both CD105+ and CD90+ cells. CD90+ and CD105+ cells each showed significantly higher sphere forming efficiency for at least 4 generations of sphere. CD90/CD105 double positive cells did not express α-smooth muscle actin (αSMA), a myofibroblast marker linked to the phenotype of BPH, in vitro and *in situ*. Similar findings were made using mesenchymal cells derived from human prostate biopsies. These data suggest that the described mesenchymal progenitor cells might provide a new target for the treatment of BPH.

Poster Board Number: F-2213

HEDGEHOG SIGNALING REGULATES MOUSE STROMAL STEM CELLS IN THE ADULT PROSTATE

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Sonic hedgehog (SHH) signaling regulates adult stem cells in self-renewing organs, including stem cells in the forebrain and hair follicle. However, it is unclear whether SHH signaling also regulates stem cells in normally quiescent organs such as the prostate. The prostate is ideal for studying adult stem cells that are normally largely dormant since they can be activated in response to tissue injury and SHH is required for regeneration. Using knock-in reporter mice, we found that SHH from basal epithelial cells signals to surrounding stromal cells based on expression of the SHH target gene Gli1. Interestingly, we identified four stromal cell types using molecular markers and cell position. Furthermore, GLI1 expression is enriched in the cell type (subepithelial cells) closest to the source of SHH. Using Genetic Inducible Fate Mapping (GIFM) with Gli1CreER/+; R26LacZ/LacZ mice to follow the fate of GLI1(+) cells *in vivo*, we uncovered that GLI1-GIFM cells have long-term self-renewal capacity since they can repopulate the stroma during multiple rounds of androgen-mediated regeneration following castration-induced involution. Notably, GLI1-GIFM cells only contribute to the stroma and not to the epithelium, suggesting that HH signaling could directly regulate stromal stem cells, and only indirectly regulate epithelial stem cells. Furthermore, analysis of the percentage of each cell type marked by GLI1-GIFM after regeneration and cell proliferation during regeneration suggests subepithelial cells are the stem/progenitor cells for mature smooth muscle cells. Taken together, our results indicate paracrine SHH signaling could have critical role in regulating stromal regeneration in the adult prostate.

Poster Board Number: F-2214

EXPANSION OF MESENCHYMAL STEM CELLS WITHIN THE MURINE BONE MARROW NICHE REQUIRES ACTIVATION OF THE FIBRINOLYTIC PATHWAY

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Mesenchymal stem cells (MSCs) hold great potential for the treatment of various degenerative diseases and immune disorders, largely because of their differentiation potential and immunoregulatory capacity. MSCs can differentiate into osteoblasts, chondrocytes and adipocytes. The bone marrow (BM) is one of the major sources of MSC. Here, we show that the serine protease tissue-type plasminogen activator (tPA) increases the number of CD45-, TER119-, Sca-1+, PDGF-Ralpha+ murine MSCs within the BM, a process that requires endogenous plasminogen (Plg) and matrix metalloproteinase-9 (MMP-9). In Plg-/- and MMP-9-/- mice, tPA-mediated MSC expansion is impaired. Importantly, kit ligand (KitL) augments the number of MSC within the BM. tPA and KitL administration induces platelet-derived growth factor (PDGF) expression on total BM cells and KitL receptor (c-Kit)+ endothelial cells, respectively. PDGF is a well-known growth factor for MSCs. These data provide novel insides into MSC biology, whereby altering the proteolytic balance promotes the cytokine bioavailability within the BM niche and MSC expansion *in vivo*.

Poster Board Number: F-2215

TRANSPLANTATION OF MESENCHYMAL STEM CELLS DERIVED FROM BONE MARROW AFTER SPINAL CORD INJURY CONTRIBUTE TO FUNCTIONAL RECOVERY AND TISSUE REGENERATION BY PROMOTING THE ALTERNATIVE PATHWAY OF MACROPHAGE ACTIVATION

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Introduction: Mesenchymal stem cells (MSC) derived from bone marrow can potentially reduce the acute inflammatory response marked by infiltration of neutrophils and macrophages, activation of glial cells, and upregulated expression of proinflammatory cytokines in spinal cord injury (SCI) and thus promote functional recovery. However, the precise mechanisms through which transplanted MSC attenuate inflammation after SCI are still unclear. Clinical and experimental SCI elicits an inflammatory response that compromises mostly macrophages. Macrophages dominate sites of SCI in which they promote both injury and repair. These divergent effects may be caused by distinct macrophage subsets,

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i.e., classically activated proinflammatory (M1 type macrophage) or alternatively activated anti-inflammatory (M2 type macrophage) cells. The present study was designed to investigate the effects of MSC transplantation with a special focus on their effect on macrophage activation after SCI. Methods: Adult Sprague-Dawley rats were subjected to T9-T10 SCI by contusion with the Infinite Horizon impactor (200kdyn), then treated 3 days later with transplantation of 1.0 x 106 PKH26-labeled human MSC into the contusion epicenter. 5 µl basal medium injected rats at 3 days after SCI were used as non-treatment groups. Localization and distribution of transplanted MSC was examined by immunofluorescence with cell markers (NeuN, RIP, GFAP, OX-42) at 1 week and 5 weeks after SCI. To evaluate the transplanted MSC effects on distinct macrophage subsets, immunofluorescence staining and flow cytometry analysis (anti-iNOS, CD16/32 for M1 type macrophage and anti-arginase-1, CD206 for M2 type macrophage) were performed at 1 week after SCI. In order to follow the progression of the SCI, samples of the lesion epicenter were stained using Luxol Fast Blue (LFB) to evaluate the degree of demyelination and myelin sparring at 5 weeks after SCI. To assess the recovery of locomotive capabilities after SCI, both group were investigated at 3 days, 1, 2, 3, 4, and 5 weeks after injury. Results: The transplanted MSC migrated within the injured spinal cord without differentiating into glial or neuronal elements. MSC transplantation was associated with marked changes in the SCI environment, with significant increases in IL-4 and IL-13 levels reductions in TNF-α and IL-6 levels. This was associated simultaneously with increased numbers of alternatively activated macrophages (M2 phenotype: arginase-1 or CD206-positive) and decreased numbers of classically activated macrophages (M1 phenotype: iNOS or CD16/32-positive). These changes were associated with functional locomotion recovery in the MSC transplanted group, which correlated with preserved axons, less scar tissue formation and increased myelin sparring. Conclusion: Our results suggested that the transplantation of MSC after SCI shifts the phenotype of macrophages after injury from classically activated macrophages (M1 phenotype) to that of alternatively activated macrophages (M2 phenotype) during the acute phase; this was associated with the presence of relevant cytokine profiles, reduction in inhibitory scar tissue/cavity formation in the subacute/chronic phase, and the provision of a permissive environment for axonal extension and functional recovery.

Poster Board Number: F-2216

MORPHOLOGY-BASED CELL QUALITY ASSESSMENT OF DIFFERENTIATION POTENTIAL OF MESENCHYMAL STEM CELLS

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Cell-morphology had long been used as an important indicator of cell quality in cell biology for the daily care of cells. However, such experience-based morphological importance has not been well examined in the aspect of automation for industrialization. For the cell therapy, such non-invasive technique to evaluate and assess the quality of cells is strongly required for its industrialization with the growth of stem cell research and its achievements. To produce intact cells for therapy, the quantitative assessment technology offers a great potential to standardize and quantify the advancing stem cell culture protocols. Human bone marrow-derived stem cells (hBMSCs) had been widely studied and applied to clinical cell therapies with their multipotency. However, it is also known that such stem cells require highly skilled cell culture to carefully maintain their undifferentiated status, and to differentiate into the objective type of cells. In our research, we introduced bioinformatic machine learning strategy in the cell image analysis to build a prediction model, which links "the cell morphology information" and "the experimentally determined differentiation results" of hBMSCs. By optimizing the effective cell culture technique for image acquisition and the image processing algorithms, we investigated the effectiveness of computational prediction model for evaluating the differentiation capacity of hBMSCs. Practically we examined to predict osteogenic, adipogenic, and chondrogenic differentiation capacity. By analyzing time-lapse phase contrast images of more than 3,000 images of the continuously passaged hBMSC (over 8 passages), the image-derived multi-parametric information of cell morphology was modeled to be linked to the experimentally defined staining results of differentiations. As a result, the cellular damage that reduced the multi-differentiation potency through continuous passages was effectively modeled, and the cell quality was able to be quantitatively predicted only from the cell images. Also from the microarray analysis, we further investigated the gene expression patterns that relate to the morphological prediction performance of differentiation status.

Poster Board Number: F-2217

PROSPECTIVELY ISOLATED PDGFRA AND SCA-1 DOUBLE POSITIVE DENTAL PULP-DERIVED MESENCHYMAL STEM CELL-LIKE CELLS HAVE DIFFERENT CHARACTERISTICS AS COMPARED TO PDGFRA AND SCA-1 DOUBLE POSITIVE BONE MARROW-DERIVED MESENCHYMAL STEM CELLS.

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It has been reported that various tissue-committed stem and progenitor cells exist in adult tissues and these cells maintain biological functions by their self-renewal capacity and differentiation potentials into various somatic cells. Bone marrow (BM) is considered to be the source of these stem and progenitor cells. The BM cells contain hematopoietic stem cells, which generate all types of blood cells, and mesenchymal stem cells (MSCs), which can differentiate into mesenchymal lineages, such as osteoblasts, chondrocytes and adipocytes. Many investigators showed that MSC-like cells exist in various tissues, such as adipose tissue, dental pulp and periodontal ligament. These MSC-like cells exhibit a variety of differentiation potentials and express specific MSC markers on their surfaces. Postnatal dental pulp tissue may contain MSC-like cells. Dental pulp stem cells (DPSCs) are considered as one of the tooth-derived stem cells. It has been reported that DPSCs can differentiate into not only odontoblasts, which are restricted to oral tissues, but also other mesenchymal lineages, such as adipocytes, osteocytes and chondrocytes. A flow cytometric analysis has revealed that MSCs express various cell surface markers, including undifferentiated cell specific markers. Several investigators have attempted to purify immature BM-MSCs using these specific MSC markers. Recently, a study reported that undifferentiated and proliferative BM-MSCs derived from mouse BM express PDGFRα and Sca-1, and these cells can be prospectively isolated by fluorescence activated cell sorting (FACS) (Morikawa S, et. al. J Exp Med 206:2483-96. 2009). BM-MSCs

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and MSC-like cells, which are derived from various tissues, are considered that these cells have similar differentiation potentials and expression patterns of cell surface markers. Therefore, the present study investigated whether DPSCs are able to prospectively isolate by FACS using MSC markers. As a result, we succeeded isolation of mouse DPSCs from mandibular incisors by FACS using MSC markers, such as PDGFRα and Sca-1. Mouse PDGFRα and Sca-1 double positive DPSCs with the expressions of odontoblast progenitor cell markers exhibited a high growth potential, and the ability to differentiate into osteocytes, chondrocytes and adipocytes. Immunofluorescence of the apical end of the mouse incisor revealed that PDGFRα$^+$ and Sca-1$^+$ cells existed at the putative dental cells-niche. Furthermore, the present study showed that dental pulp-derived PDGFRα$^+$, Sca-1$^+$ cells expressed CD49f and CD71, while BM-derived PDGFRα$^+$, Sca-1$^+$ cells did not express these markers. The expression levels of CD90, CD105 and PDGFRβ were different between DPSCs and BM-MSCs, which were positive for PDGFRα and Sca-1 expressions. These cells were also different in fibroblastic-colony forming activity and alkaline phosphatase activity in primary cultures. These observations suggest that the MSC-like cells are specialized in individual tissues, while the cells still retain the original cell properties, such as the potential to differentiate into mesenchymal cell lineages and the expression of the common cell surface markers. Further investigations of DPSCs may contribute to the field of regenerative therapeutics for tissue/organ repair in the near future.

Poster Board Number: F-2218

GENETICALLY ENGINEERED MESENCHYMAL STEM CELLS WITH V-ERB-A ERYTHROBLASTIC LEUKEMIA VIRAL ONCOGENE HOMOLOG 4 (ERBB4) IMPROVE CARDIAC PROTECTIVE POTENCY

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Background: Among several cell types used in stem cell therapy, mesenchymal stem cell (MSC) is a promising cell type for the treatment of myocardial ischemia in both rodent model and human clinical trials. However, poor cell viability associate with transplantation in the ischemic myocardium seemed to be a substantial impediment of its overall restorative capacity. In this study, we postulated that the overexpression of v-erb-a erythroblastic leukemia viral oncogene homolog 4(ERBB4) in mesenchymal stem cells would enhance recruitment and penetration of the transplanted cells into ischemic myocardium, leading to cardiac protection after myocardial infarction(MI). Methodology/PrincipalFindings: First, we isolated mouse MSCs by flushing the marrow of both the tibia and the femur. The cells were kept in culture till passage 6, and then a subset of MSCs were used for characterization. These spindle-shaped MSCs were positive for CD44, CD90.2, SCA-1, and negative for the hematopoietic cell lineage-speci[[Unsupported Character - ﬁ]]c antigens CD34, CD45, C-kit. Also, they could be readily differentiated into adipocyte, chondrocyte and osteocyte under relative induction medium. Then these MSCs were genetically engineered using *ex vivo* lentiviral transduction to overexpress ERBB4. The expression plasmid was labled by GFP so the modified MSCs could be easily tracked after transplantation in vivo. After optimization and multiple infections, flurescence could be observed in 80% to 90% MSCs. These manipulated MSCs were injected in MI model either overexpressing empty vector or overexpressing ERBB4 as compared groups. One month after surgical MI and MSCs injection, heart function were evaluated by echocardiography and pressure volume loop assessments in following groups: 1). MSCs overexpressing empty vector;2). MSCs overexpressing ERBB4 ;3) Placebo (saline) ;and 4) normal control. The data showed the modified ERBB4-modified MSCs can improve cardiac performance after injury. For in vitro study, , the cells were kept culturing in hypoxic chamber to mimic the environment of myocardial infarction. The preliminary data showed that the apoptosis rate of the MSCs with ERBB4 expression was lower than the MSCs with empty vector after 6h and 24h. Conclusion: Our preliminary data showed that genetically modified mouse MSCs might modulate the function of the ischemic cardiomyocyte by inhibiting apotosis, while the mechanism of their beneficial effects needs to be further investigated.

Poster Board Number: F-2219

APPLICATION OF KAPLAN MEIER ANALYSIS TO QUANTIFY CELL DEVELOPMENTAL DYNAMICS FROM SINGLE CELL OUTCOMES

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Kaplan Meier (KM) analysis is a well-established statistical method to estimate a probability survival function from lifetime data and is commonly applied to clinical trials that evaluate patient responses to treatment. However this method has not been generally applied to study single cell fate outcomes. Here we apply the KM estimator to quantify the empirical probability of single cell fate outcomes in culture as observed by time lapse microscopy. This method of analysis was evaluated using long-term cell lineage tracking data. The *in vitro* development of cardiac colony forming units fibroblast (cCFU-F) or granulocyte macrophage progenitors (GMP) were characterised by observing the time of a) mitosis and b) apoptosis and c) fluorescent reporter gene expression in consecutive progeny. KM analysis was performed separately on cell groups classified according to their culture environment and phenotype. The empirical probability distribution function for these events was conditionally dependent on cell class, and closely resembled lag exponential distributions. Therefore cell class specific rates for differentiation, apoptosis and lineage commitment could be estimated from KM curves. Cell developmental dynamics were simulated at the single cell pedigree and population levels with a multi-type Smith Martin model using the estimated cell class specific rates for differentiation, apoptosis and lineage commitment. Closer examination of lineage pedigrees showed that cell classes were not homogeneous; there was lower heterogeneity for cells within pedigrees that comprise the cell class and cells within a pedigree did not develop independently from one another. For example cell generation times were strongly correlated in sister cells. There was vertical transmission of generation times, particular if mothers had a short G1 phase. Furthermore in the GMP system, macrophage progenitor commitment within a clone was characterised by synchronous expression of lysozyme GFP and abrupt prolongation of cell cycle, suggesting that the haematopoietic lineage program is more deterministic at the clonal level. Therefore the stochastic (exponential) cell fate outcomes observed at the population level is attributed to both intra- and inter-clonal diversity. Future work will aim to develop methods for high throughput lineage tracking by live cell imaging, and to understand the role played by extracellular signals directing the dynamics of differentiation and proliferation. An understanding of stem and progenitor cell developmental dynamics is required for clinical translation of stem cell sciences.

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Poster Board Number: F-2220

IN VIVO TRACKING OF THE ROLE OF ENDOGENOUS BONE MARROW MESENCHYMAL STEM CELLS

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Mesenchymal cells are present in most tissues and of particular importance in the regenerative process of bone and bone marrow. Despite their pivotal roles in the maintenance of bones and hematopoiesis, the in vivo origin, lifespan, and dynamics of these cells are poorly understood. While putative mesenchymal stem cells (MSCs) have been defined by in vitro multi-lineage potential and immunophenotype, and are in clinical testing, how such cells perform in vivo remains a matter of debate. Using genetically engineered mice in combination with intravital microscopy, we found that mature osteoblasts have a relatively short lifespan and that they do not proliferate even after injury. Rather, they are continually replenished by progenitor cells with an immunophenotype and in vitro functions consistent with that of mesenchymal stem cells. These MSC-like cells have a perivascular origin and efficiently labeled by Mx1 (myxovirus resistance-1). Further, they overlap with a subset of perivascular nestin+ MSCs. These cells maintain long-term repopulation of osteoblasts in vivo, respond to tissue stress, migrate to sites of injury, and supply the majority of new osteoblasts in fracture healing. Single cell transplantation yielded progeny that both preserve progenitor function and differentiate into osteoblasts producing new bone. Additionally, they are capable of both local and systemic translocation and serial transplantation. While these cells meet the definition of MSC in vitro in that they can undergo single cell-derived multi-lineage differentiation, they are osteo-lineage restricted in vivo and do not appear to participate in the generation of chondrocytes, skeletal muscle cells, endothelial cells or fibroblasts in growing and adult animals. Therefore, *ex vivo* multipotency of MSC should not be assumed to indicate in vivo function and the commonly defined, bone marrow derived MSC may be a heterogeneous population with the population defined here representing a highly dynamic and stress-responsive stem/progenitor cell population with fate-restricted potential in vivo.

Technologies for Stem Cell Research

Poster Board Number: F-2222

DERIVATION OF NANOG KNOCK-IN GFP REPORTER IPS CELL LINES USING MEGANUCLEASE-INDUCED HOMOLOGOUS RECOMBINATION

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NANOG is a critical transcriptional factor for regulating pluripotency, and it is specifically expressed in the inner cell mass, ES cells, and fully-reprogrammed iPS cells. The purpose of this study is to generate NANOG-GFP reporter iPS cell lines to visualize the NANOG gene expression. Such cells would be powerful tools to monitor NANOG for assessing pluripotency, differentiation potentials, and reprogramming mechanisms. Homologous recombination (HR) is the method of choice to develop such reporter cells, since it will allow the integration of a single copy of a reporter at a specific locus. Such integration can be achieved using a homing endonuclease, called meganuclease, which specifically recognize a sequence of 12 to 30 base pairs of genomic DNA to induce a double strand break at the desired locus. While the double strand break is repaired by host cell, by transfecting a repair matrix with a GFP reporter, homologous recombination takes place within the cell and allows integration of the reporter gene into the desired locus. Here, we designed and generated a meganuclease targeting the intron 1 of human NANOG gene locus, as well as unique repair matrices, to facilitate introduction of the GFP gene at the NANOG start codon, to allow GFP expression under the control of the endogeneous NANOG promoter. To verify the strategy, CHO and 293H cells were transfected with the meganuclease and confirmed the enzymatic activity. Then, we succeeded to knock-in the GFP gene at the NANOG exon 1 locus in 293H cells. In parallel, the expression of NANOG meganuclease was demonstrated in human iPS cells using similar methodologies and intermediate levels of targeted mutagenesis were observed using an assay based on Non-Homologous End Joining (NHEJ) repair mechanisms and deep sequencing. Experiments were performed in iPS cells to evaluate the knock-in of GFP into the NANOG exon 1 gene by using an optimized protocol of the meganuclease-mediated HR. NANOG-GFP reporter iPS cell lines will allow us to assess NANOG promoter activation and silencing by measuring GFP expression, during iPS cell differentiation and reprogramming studies. This should help better understand the mechanisms involved in the induction of pluripotency.

Poster Board Number: F-2223

GENOME ENGINEERING OF IPS CELLS USING MEGANUCLEASES AND TALENS

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Induced pluripotent stem cells (iPS) can be derived from adult somatic cells with various genotypes and phenotypes and have the capability to proliferate indefinitely and to differentiate into any human cell type. These characteristics make iPS cells ideal candidates for improvements to current drug screening approaches and for the development of novel regenerative medicines. Currently available differentiation protocols are frequently incomplete and do not always reach the desired terminal and/or mature stages. Genome engineering techniques based on homologous recombination (HR) represent a powerful means to improve these differentiation processes, by allowing controlled expression of defined factors, along differentiation pathways, leading to improved terminal maturation and homogeneity of cells. However, HR is a precise, but inefficient mechanism, especially in iPS cells. Nevertheless, efficiency can be significantly improved by combining this approach with selectively cutting endonucleases, such as meganucleases and TAL effectors nucleases (TALENs), which induce a DNA double strand break at a defined locus within the genome. These endonucleases can be designed to target a unique locus in a safe harbour (SH) region of the genome. The advantage of this approach is that unlike random integration, where gene insertion can interfere with endogenous gene activity, transgenes can be integrated in a predictable and reproducible manner whilst maintaining the genome integrity of

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the cell. In this study, several meganucleases and TALENs, targeting SH loci in the iPS genome, as well as associated unique GFP or Luciferase repair matrices, were designed and produced. Initially, endonuclease activity was confirmed in CHO and 293H cell lines. Successful knock-in was also demonstrated in 293H cells and the most efficient endonucleases were then assessed in iPS cells. Globally, endonucleases were well expressed in iPS cells, but dose response experiments highlighted toxicity effects at high concentrations for some of them. Despite similar expression profiles, and depending of meganucleases/TALENs assessed, different levels of activity were measured through targeted mutagenesis experiments. Selected endonucleases were then evaluated for knock-in efficiency in iPS cells. Successful targeted genome integration was identified for specific loci, demonstrating the value of this technology to improve HR and facilitate genome engineering in iPS cells. Next steps will be to use these newly defined meganucleases and TALEN-based tools to generate engineered iPS cell lines with the potential to express critical differentiation factors. Development of relevant iPS-derived cellular models available both for drug screening and regenerative medicine will be achieved by modifying the time, level and duration of factor expression during the differentiation process.

Poster Board Number: F-2224

NERVE FORMATION IN 3D HISTOCULTURE FROM HAIR FOLLICLE NESTIN-EXPRESSING STEM CELLS

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We have previously discovered nestin-expressing multipotent hair follicle stem cells (Proc. Natl. Acad. Sci. USA 100, 9958-9961, 2003; Cancer Res. 65, 2337-2343. 2005; Cancer Res. 65, 5352-5357, 2005; Cell Cycle 7, 1865-1869, 2008). Mouse vibrissa (whisker) hair follicles were obtained from transgenic mice, in which the nestin promoter drives the expression of green fluorescent protein (ND-GFP). The whisker follicles contain nestin-expressing stem cells which originate from the follicle bulge. The whiskers were placed in long-term 3-D histoculture on Gelfoam® and imaged with confocal microscopy. Nestin-GFP expressing cells were observed to traffic from the bulge area (BA) of the histocultured whisker and proliferated at the cut end of the severed nerve to extend the nerve which contained nestin-expressing spindle-shaped cells that co-expressed β3-tubulin as well as nestin-negative round cells that expressed GFAP. In subsequent experiments, whisker follicles with a growing sensory nerve, expressing ND-GFP, were co-cultured on Gelfoam® with the trigeminal nerve derived from mice expressing red fluorescent protein (RFP). Whiskers with a growing ND-GFP expressing nerve were placed in Gelfoam® culture next to the trigeminal nerve expressing RFP. On day 6 of Gelfoam® culture, dual-color fluorescence confocal imaging showed that the whisker-follicle sensory nerve grew out and fused with the trigeminal nerve. Our results suggest a major function of the nestin-expressing stem cells in the hair follicle may be formation of the hair follicle sensory nerve which can then fuse with the trigeminal nerve, which may mimic the *in vivo* sensory circuit. Gelfoam® histoculture is a very powerful method to study stem cell behavior of the hair follicle including nerve formation and the development of a sensory circuit.

Poster Board Number: F-2225

CONFOCAL IMAGING OF BEHAVIOR OF NESTIN-EPXRESSING MULTIPOTENT STEM CELLS IN LONG-TERM 3D HISTOCULTURE

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We have previously discovered that nestin-expressing multipotent hair follicle stem cells are located above the hair follicle bulge and can differentiate into neurons and other cell types *in vitro* (Proc. Natl. Acad. Sci. USA 100, 9958-9961, 2003; Proc. Natl. Acad. Sci. USA 102, 5530-5534, 2005). The nestin-expressing hair follicle stem cells can differentiate into Schwann cells and promote the recovery of pre-existing axons when they were transplanted to the severed sciatic nerve or spinal cord (Proc. Natl. Acad. Sci. USA 102, 17734-17738, 2005; Cell Cycle 7, 1865-1869, 2008). We have also demonstrated that the whisker hair follicle contains nestin-expressing stem cells in the dermal papilla (DP) as well as in the bulge area (BA) (Cell Cycle 10:830-839, 2011) but that their origin is in the BA (J. Cell. Biochem. 112, 2046-2050, 2011). In order to image trafficking and other behavior of nestin-expressing stem cells in the whisker, we cultured whiskers from transgenic mice expressing nestin-driven GFP (ND-GFP) in 3-dimensional histoculture on Gelfoam®. Confocal microscopy was used in order to image the nestin-expressing stem cells whiskers extracted from transgenic mice expressing ND-GFP. We were able to culture the whiskers for at least 2 weeks on Gelfoam®, during which time it was observed that ND-GFP-expressing stem cells in the histocultured whiskers trafficked from the bulge area (BA) toward the dermal papilla (DP) area and extensively grew out onto Gelfoam®. This new method of long-histoculture of whiskers from ND-GFP mice will enable the extensive study of the behavior of nestin-expressing multipotent stem cells of the hair follicle.

Poster Board Number: F-2226

CHEMICALLY DEFINED HUMAN RECOMBINANT LAMININS PROVIDE UNIQUE AND BIORELEVANT SUBSTRATES FOR CULTURE OF HUMAN ES, IPS AND DIFFERENTIATED CELLS

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Establishment of a chemically defined, xeno-free and feeder-free environment for culturing human pluripotent cells has been a major, long withstanding goal in the field of regenerative stem cell therapy. Pluripotent human embryonic stem (ES) cells and induced pluripotent stem (iPS) cells have in vitro been difficult to maintain. There are also difficulties to keeping differentiated cells alive maintaining their phenotype. Human ES and iPS cells differentiate, differentiated cells de-differentiate, die or change functional characteristics. The solution for these problems can likely be found in *in vivo* biology. Extracellular matrix proteins are an important part of *in vivo* niches and have been shown to influence cell differentiation, adhesion, proliferation, migration, phenotype maintenance and self-renewal of many types of cells, including stem cells. Laminins, the main component of the basement membrane, are a family of heterotrimeric glycoproteins composed of α, β and γ chains that exist as five, three and three genetically distinct types forming 16 different combinations in human tissues. Laminins bind to both the extracellular collagen and integrin receptors on the cell surface.

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It has been shown that the integrin receptors can activate various cell signaling pathways giving laminins themselves growth factor like properties. Laminins are the crucial link between cells and organs and the extracellular matrix between the organs. Even though there are many different proteins in the basement membrane, the laminins are the only ones expressed tissue-specifically. Laminin-511 and 521 are expressed early in embryogenesis, while pancreatic cells produce laminin-511 and 411 and this specificity gives the laminins a role in creating the cellular niches. For instance, on top of laminin-511, the human ES cells self-renew with normal karyotype for at least 4 months (20 passages), and form teratomas with components of all three germ layers after injection into immundeficient mice. Laminin-521 facilitates single human ES and iPS cell survival, rapid monolayer proliferation with maintained pluripotency and karyotypic stability for months after repeated single-cell suspension passaging without ROCK inhibitor. Therefore it is a major advantage to use biologically relevant laminins for in vitro cultures of both human pluripotent stem and differentiated cells, in order to ensure optimal survival, maintenance and differentiation of correct, therapeutic cellular phenotypes. Tissue-purified laminins have been available for years. However, since laminins are extremely large and complex proteins that are, due to high cross-linking, very difficult or virtually impossible to isolate in native forms from tissues, current strategies for isolating laminins from tissues, like placenta, cause protein degradation, lot-to-lot variation that results in variable and unreliable research results. Only recently, techniques have been developed to manufacture human recombinant laminins from transfected human cell lines. Human recombinant laminins are both chemically defined and xeno-free and have been used widely in culturing human ES and iPS cells, and some differentiated cell types. Recombinant laminins are the only matrices available that are defined, xenofree and provide biological niches for various cells.

Poster Board Number: F-2227

PROTEOMIC ANALYSIS OF MICROVESICLES DERIVED FROM HUMAN MESENCHYMAL STEM CELLS

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Mesenchymal stem cells (MSCs) have emerged as one of the most promising stem cells for treating various degenerative or incurable diseases. Recent studies have shown that microvesicles (MVs) from MSCs contribute to the recovery of damaged tissues in different animal disease models. Here, we profiled the MV proteome to elucidate the potential contribution of MVs to MSC-mediated cellular processes associated with their therapeutic effects. MVs purified by a combination of sucrose gradient and Optiprep density gradient centrifugation were analyzed by 1-D SDS PAGE and nano-LC-MS/MS. A total of 730 microvesicular proteins were identified with high confidence from three independent experiments. By integrating protein-protein and protein-DNA interactions, we found that a substantial portion of the MV proteome could be regulated by pluripotency inducing transcription factors (TFs), and that they could also closely interact with self-renewal and differentiation related proteins when mRNA data from undifferentiated (embryonic stem cells, ESC) and differentiated cells (mouse embryonic fibroblast cells) were further integrated. Functional enrichment analysis revealed that the processes enriched by the MV proteins were largely overlapped with those enriched by the up-regulated genes in ESC, including cell adhesion, migration, and blood vessel development. Overall, these results indicate that a large number of MV proteins are associated with self-renewal and differentiation. Collectively, we identified a large spectrum of MV proteins derived from MSCs that have the potential to act as mediators of cell-cell communication and/or paracrine modulators of tissue repair and regeneration in diseases. *This study was supported by grant SC-1110 , SC-2130 and MEST(2010-0020353) from the Stem Cell Research Center of the 21st Century Frontier Research Program, the Ministry of Education, Science, and Technology of the Republic of Korea*

Poster Board Number: F-2228

DERIVATION OF HUMAN EMBRYONIC STEM CELLS FROM DISCARDED IMMATURE OOCYTES

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Human embryonic stem cells (ESCs) are routinely derived from "surplus" embryos donated by patients undergoing infertility treatments by *in vitro* fertilization (IVF). During conventional IVF procedures, a small number of immature oocytes are recovered that are routinely discarded. We sought to investigate possibility of rescuing such oocytes by *in vitro* maturation (IVM) and their potential to develop to blastocysts and ESCs following *in vitro* fertilization. We approached 55 women undergoing IVF treatment at Oregon Health & Science University in 2011 to participate in this study. Of these invited patients, 50 agreed and consented to donate their immature oocytes for stem cell research (91% consent rate). Following gonadotropin stimulation, hCG-priming, and oocyte retrieval, 75 germinal vesicle (GV) and 42 metaphase I (MI) stage immature oocytes were harvested from 36 women and donated for this study. Following IVM using Sage medium, 67% (50/75) of GV and 69% (29/42) of MI oocytes matured to the metaphase II (MII) oocytes as judged by extrusion of the first polar body (PB). Fifty-eight MII oocytes were selected for fertilization with donor sperm using intracytoplasmic sperm injection (ICSI). Based on pronuclear formation, 33 (57%) oocytes were successfully fertilized and following *in vitro* culture only 4 (12%) of fertilized oocytes developed to blastocysts. Interestingly, all developed blastocysts were from GV matured oocytes. We plated these blastocysts on feeder layers in standard ESC culture medium consisting of DMEM/F12 medium supplemented with 0.1mM nonessential amino acids, 1 mM l-glutamine, 0.1 mM β-mercaptoethanol, 5 ng/ml basic fibroblast growth factor, 10% fetal bovine serum and 10% knockout serum replacement. Following manual isolation of initial colonies and further passaging, two stable ESC lines were established (50% isolation rate). Our results suggest that immature human oocytes donated during IVF cycles can be efficiently matured and fertilized. However, developmental potential to blastocysts is significantly compromised. Nevertheless, blastocysts produced from IVM oocytes supported high ESC derivation rates. This study was approved by the OHSU institutional review board (IRB #6709) and supported by institutional funds and the Center for Women's Health Circle of Giving grant.

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Poster Board Number: F-2229

IDENTIFICATION OF CELL SURFACE MARKERS FOR EFFECTIVE ANALYSIS AND ISOLATION OF HUMAN INDUCED PLURIPOTENT STEM CELLS BY FLOW CYTOMETRY

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Human induced pluripotent stem cells (hiPSCs) hold enormous potential for industrial and therapeutic use, however several obstacles impede the scalable production, characterization and expansion of hiPSC clones. Fluorescence activated cell sorting (FACS) is a powerful approach for selection of cells with preferred characteristics, however human pluripotent stem cells have dramatically reduced viability when dissociated into the required single cell suspension. Moreover, cell surface signatures that can be used to distinguish bona fide hiPSC from partially reprogrammed cells by FACS remain elusive. We previously described the identification of a small molecule media additive SMC4™ to support the viability of single cell dissociated hiPSCs and the subsequent development of a platform for the high-throughput generation and characterization of hiPSC clones using FACS. We have further refined this platform by employing previously described markers $CD13^-/SSEA4^+/Tra160^+$ in our cell sorting strategy for the isolation of hiPSC clones. In an enhanced sorting strategy, actively reprogramming pools of fibroblast origin are initially depleted of the fibroblast cells using CD13 negative selection followed by an enrichment step employing hiPSC surface makers SSEA4 and Tra160. By using $CD13^-/SSEA4^+/Tra160^+$ selection criteria, individual hiPSCs are readily identified during the reprogramming process and can be directly plated into feeder free 96-well plates at clonal density to deliver hiPSC clones in an efficient and high-throughput manner. Furthermore, a survey of embryonic stem cell markers revealed that CD30 and CD50 may add an additional layer of selection criteria for the identification of bona fide hiPSCs. Preliminary data suggest that CD30 may better distinguish clones that express the core pluripotency genes and potentially serve as a surrogate marker for Nanog. The combination of SMC4™ culture along with $CD13^-/SSEA4^+/Tra160^+/CD30^+$ selection may represent a more stringent and highly efficient method of identifying and isolating hiPSCs from a heterogeneous pool of reprogramming cells. These methods will further enable the high throughput generation of hiPSC clones for pharmaceutical applications.

Poster Board Number: F-2230

HIGH-THROUGHPUT IDENTIFICATION AND SUBSEQUENT PRODUCT DEVELOPMENT OF THERMALLY-RESPONSIVE SYNTHETIC POLYMER HYDROGELS FOR ENZYME FREE PASSAGING AND LONG TERM MAINTENANCE OF HUMAN EMBRYONIC STEM CELLS

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Human embryonic stem cell culture environments have generally relied on the provision of recombinant or purified extracellular protein matrices to support attachment and growth. Here we report the high-throughput identification and subsequent product development of synthetic polymer hydrogels as chemically defined and protein-free alternatives to purified or recombinant extracellular matrixes for hESC attachment and long maintenance, with enzyme free passaging abilities. On the synthetic polymer hydrogels and in a defined medium (mTESR) different hES cell lines maintained their undifferentiated state for over 20 passages, while maintaining their pluripotent nature. Critically, we have shown that the selected hydrogels can be scaled-up and efficiently coated onto standard multi-well plates allowing this groundbreaking technology to be made commercially available in established formats to external stem cell researchers. These substrates constitute a paradigm shift in stem cell culture technology, obviating reliance on both animal sourced or recombinant protein substrates and enzymatic or chemical dissociation for passaging which should facilitate both new advances in the field of stem cell research and support the development of novel therapies.

Poster Board Number: F-2231

EFFECT OF HYPOXIA ON *EX VIVO* EXPANSION OF HUMAN MESENCHYMAL STEM CELLS

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Background In vitro atmospheric oxygen pressure is around 150 mm Hg (20% O2), whereas physiologic (in vivo) oxygen pressure ranges between 50 and 5 mm Hg (7%-0.7% O2). The normoxic environment in cell culture does not refer to a physiological stem cell niche. The aim of this study was to investigate the effect of oxygen concentration on human mesenchymal cell expansion. We analyzed human voided urine stem cells (hVUSCs), upper urinary track urine stem cells (hUUSCs), amniotic fluid stem cells (hAFSCs), dental pulp stem cells (hDPSCs) and umbilical vein endothelial cells (HUVECs) to achieve clinical relevant numbers and retention of stem cell properties. Method hVUSCs, hUUSCs, hAFSCs, hDPSCs and HUVECs were cultured under 5% O2 hypoxic or 20% O2 normoxic condition for 5 days. For proliferation rate evaluation, cell counting kit-8 assay and cell cycle analysis were performed. Effect of hypoxia on the cell senescence and apoptosis was evaluated with β-galactosidase staining and TUNEL assay. Karyotyping was performed to evaluate chromosomal stability. Immunophenotype analysis was performed with Flow cytometry. The *ex vivo* expanded cells were induced into osteogenic, adipogenic or chondrogenic cells for analysis of multi-lineage differentiation efficiency. The molecular variation was analyzed with the expression of stem cell markers (Oct4, C-Myc and Nanog) and hypoxia-inducible marker (HIF-1α) by real-time PCR. Bone marrow stromal cells (BMSCs) and Jurkat cells were used for controls. Result hVUSCs, hUUSCs, hAFSCs and hDPSCs cultured under hypoxia were showed significantly increased proliferation rate and S-phase cells compared to those cultured under normoxic condition. Senescence and apoptosis was inhibited under hypoxia. The chromosomal stability was maintained during expansion under hypoxia. In immunophenotype analysis, hypoxia cultured cells were sustained the high level of mesenchymal stem cell surface markers. Hypoxic condition enhanced the multi-lineage differentiation efficiency of cells compare to the normoxia cultured cells. In real-time PCR assay, the hypoxia cultured cells expressed the high level of stem cell markers and HIF-1α. In case of HUVECs, hypoxic condition does not affect cell expansion on *ex vivo* culture. Conclusion The 5% O2 hypoxic condition enhances cell proliferation rate and retention of stem cell properties in hVUSCs, hUUSCs, hAFSCs and hDPSCs compared to 20% O2 normoxia.

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Poster Board Number: F-2232

IDENTIFICATION OF HUMAN STEM CELL REGULATORS THROUGH HIGH-THROUGHPUT FUNCTIONAL SCREENS

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The blood system is organized as a hierarchy sustained by stem cells at the apex. Hematopoietic stem cells (HSCs) give rise to proliferative progenitors which in turn are responsible for the output an estimated 1x10^12 differentiated cells per day. The unique potential of HSCs to renew the blood system throughout the lifetime of an individual has been the subject of intense investigation. Although murine models have revealed important circuitry that underlies HSC self-renewal, an understanding of the molecular components that sustain HSCs in humans is very limited. We developed a high-throughput approach to asses candidate stem cell regulatory genes *in vivo* using the xenograft assay, which is the only reliable means by which human HSC function can be read out. Candidate genes were selected from expression arrays comparing primitive versus more differentiated cord blood and leukemia cell populations. 96 overexpression lentiviruses were created and mixed in pools of 8-12 lentiviruses. These pools were used to transduce human cord blood cells, which were then injected into immunocompromised mice to determine if HSC output was altered. After 16 weeks of *in vivo* competition, the abundance of every gene was assessed by quantitative PCR and compared to the cells before injection. We discovered 4 genes that significantly enhance HSC output. These validated candidates include the transcriptional inhibitor ID2, which has previously been shown to disrupt the myeloid/lymphoid bifurcation step. We found a striking regulation of human HSC pool size and phenotype by over-expression of the transcriptional inhibitor ID2 *in vivo*. Taken together, by applying an *in vivo* high-throughput screen to primary human cord blood cells, we have uncovered several new regulators of human HSC function. Currently, our studies are focused on mechanistic insight into how these HSC regulatory genes modulate transcriptional programs, cytokine balance and stress response. By gaining a thorough understanding of the signals that maintain HSCs, we are working towards drawing a complete wiring diagram to represent the HSC state.

Poster Board Number: F-2233

GENETIC STABILITY OF HUMAN MESENCHYMAL STEM CELLS EXPANDED USING THE QUANTUM CELL EXPANSION SYSTEM

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Quantum Cell Expansion System (Quantum) is a novel hollow fiber-based device from TerumoBCT that automates and closes the cell culture process, dramatically reducing labor intensive processes such as manual cell culture feeding and harvesting. Manual flask and cell factory processes for the production of clinical-scale quantities of human bone marrow-derived mesenchymal stem cells (hMSC) have been successfully translated onto the Quantum platform. The translation of this formerly static manual process performed primarily on tissue culture polystyrene (TCPS) substrates may raise the question of whether hMSC cultured on a hollow fiber platform yields comparable cell quality. To this end, TerumoBCT has carried out a rigorous battery of assays to determine the genetic stability of MSC produced with the Quantum System. The concept of genetic stability can be very encompassing. Many factors can influence the types of biological testing required to show genetic stability depending on the cell type and end-use of the product harvested from the device. Genetic stability was assessed by phenotypic characterization, micronucleus formation, morphology, potency (trilineage differentiation capacity and growth kinetics), tumorigenic potential (xenograft; "Nude" mice model), and chromosomal characterization (Spectral Karyotyping "SKY" analysis) to determine the presence of chromosomal aberrations in the cell population. HMSCs expanded exclusively in T225 culture flasks were evaluated using the same battery of tests performed using hMSCs expanded in the Quantum. Whole Bone Marrow (WBM) was directly loaded into Quantum and hMSCs were isolated by adherence in the bioreactor of the Quantum system. HMSCs were expanded, harvested by trypsinization, and reloaded into a new Quantum five (5) times per donor (three (3) donors). Spectral Karyotyping and the xenograft were performed using cells harvested from Passage 2. Human Mesenchymal Stem Cells harvested from Quantum demonstrated phenotype, morphology, and trilineage differentiation capacity characteristics consistent with the International Society of Cell Therapy (ISCT) standard for hMSCs. The cell population showed no tumor formation in athymic mice 60 days post-transplant, no chromosomal aberrations, and no DNA damage as measured by increased levels of micronucleus formation. Therefore it can be concluded that Quantum-produced hMSC are of comparable quality and demonstrate analogous genetic stability to hMSC cultured on TCPS.

Poster Board Number: F-2234

HIGH-THROUGHPUT CHARACTERIZATION USING FLUORESCENCE CELL BARCODING OF HUMAN IPSC AND IPSC NEURONAL DERIVATIVES

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Induced pluripotent stem cell (iPSC) technology has opened the door for modeling human disease in the relevant cell types and in the genetic background of interest. This is particularly compelling for the study of sporadic disease where we do not know the genetic contribution. Elucidating how genomic variation drives sporadic disease will require iPSC lines from many human patients. However, one of the current limitations in using iPSC for large scale modeling of sporadic disease, is the expensive and time consuming methods used in iPSC generation and characterization. In addition, there is a lack of high-throughput assays that will allow for phenotypic characterization of iPSC derivatives in a time and cost-effective manner. For example, in sporadic Alzheimer's disease, there are numerous reports of abnormal endocytic and lysosomal systems, but immunofluorescence staining and the following microscopy is slow and expensive. Therefore, we have adapted a flow cytometry technique called Fluorescent Cell Barcoding (FCB) to rapidly characterize and identify iPSC with high expression of pluripotency markers and developed assays for high-throughput phenotypic characterization of neurons and other iPSC derivatives. We report that this technique is high-throughput, rapid and reduces cost over current methods more than 50-fold. The FCB method will allow for large-scale reprogramming of human cells to model sporadic disease and to characterize phenotypes of iPSC derivatives.

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Poster Board Number: F-2235

GENERATION AND VALIDATION OF MONOCLONAL ANTIBODIES TO 17 NEWLY IDENTIFIED CELL SURFACE PROTEINS THAT ALLOW ENRICHMENT OF HUMAN PLURIPOTENT STEM CELLS.

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The ability of human embryonic stem (hES) cells and human induced pluripotent stem (hiPS) cells, collectively termed human pluripotent stem cells (hPSCs), to indefinitely self-renew and to differentiate into essentially all cell types renders them potential sources of material for a wide range of clinical applications. However, there remains a real need to develop new cellular markers that will allow both large scale purification of live hPSC cultures, as inputs to differentiation assays, and identification and removal of unwanted residual tumorigenic pluripotent cells from hPSC-derived cell populations following differentiation. We are generating and characterising antibodies that recognise novel hPSC-associated epitopes that will be useful for both the enrichment and removal of live OCT3/4 expressing cells. We have previously reported the use of a FACS-based immunotranscriptional profiling system to characterise multiple hPSC lines (both hES and hiPS), and the subsequent identification of the subpopulation of hPSC that expresses high levels of the pluripotency-associated cell surface antigens CD9 and GCTM-2. Combined with membrane polysome translation state analysis, these data have identified all proteins likely to be expressed on the surface of pluripotent cells, leading us to a final selection of known genes for candidate cell surface proteins, which had not been previously associated with pluripotent cells, but which we observed were switched off very rapidly upon differentiation. We have subsequently generated antigens for 35 of these candidate cell surface proteins for which commercially available antibodies were either not available or did not detect epitopes on live hPSC by FACS analysis. These antigens have been generated via peptide synthesis or protein expression in both prokaryote and eukaryote cells. Following immunisation, confirmation by direct antigen array analysis and high throughput FACS screening of hybridomas, to date we have identified and expanded ~200 novel hybridomas of interest. We have now successfully subcloned ~80 IgG monoclonal antibodies (MAbs) that recognise 17 novel pluripotency-associated epitopes which are detected on live hPSCs by flow cytometry analysis. Hybridomas that bind to these 17 hPSC epitopes of interest have been grown to scale in reduced serum conditions and MAbs isolated on Protein A/G columns, followed by size exclusion chromatography and centrifugal concentration. We present here the novel hPSC-associated proteins for which we have generated and validated purified MAbs. Characterisation studies on two hES and two hiPS cell lines by multiparameter immunostaining and FACS analyses demonstrated the correlation of each MAb hPSC profile against our established $CD9^{hi}$/GCTM-2^{hi}/$OCT3/4^{hi}$ profile. Time course differentiation studies and subsequent FACS profiling in conjunction with OCT3/4 expression are also in progress for each MAb. In further studies, these novel hPSC-identifying MAbs are being coupled to magnetic beads and to toxins for testing in our hPSC purification and off-target cell purging assays (see related abstract: Yu-Chieh Wang et al). We anticipate that these antibodies will be extremely useful for the identification, enrichment and removal of hPSCs and moreover, that they will enable further exploration of the relationship between the proteins detected by these antibodies and the maintenance and/or loss of pluripotency.

Poster Board Number: F-2236

HUMAN EMBRYOID BODIES AS 3D *IN VITRO* MODEL FOR DEVELOPMENT TOXOCITY PREDICTION

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For pharmaceutical companies, defining relevant and accurate protocols for toxicity prediction is a key step in order to avoid drug development failure at clinical phases. Due to the complexity of the processes involved in the development of the embryo, chemical development toxicity is currently mainly based on animal testing. On top of being expensive and time consuming, these experiments require the sacrifice of large number of animals. Moreover, it has been proven that the animal model cannot be considered as relevant enough for teratogenicity prediction. In such context the human embryonic stem cells (hESC) *in vitro* model represents an important opportunity for developmental toxicity prediction. Indeed, hESC are pluripotent and can recapitulate *in vitro* the early stages of the embryo development. Testing drugs on such cell culture open a new way of defining toxicity. Indeed, it appears that most of the drug influencing the differentiation (without being cytotoxic) correlate drugs classify as teratogen by the Food and Drug Administration. Here we show a complex and accurate protocol for developmental toxicity in a High Content Screening (HCS) context. We have already successfully performed a large scale compound image based screening to identify small molecules inducing differentiation and correlate our results with teratogen agents. To increase the prediction quality, we performed with a 3 dimensional (3D) Embryonic Bodies (EB), instead of using a convenient 2D monolayer cell culture. EB is 3D cell mass, mimicking the early stage of the embryo development; therefore it is more feasible for evaluation of potential developmental toxicity. To validate our protocol hESC was used to overcome misjudgment which is can be caused by using mESC. We tested three categories of well-known drugs, strongly teratogen, weak teratogen, none teratogen. For each category, we selected three drugs: hydoxyurea, cyclophosphamide, and isotretinoin (accutane) for strongly teratogen; dexamethasone, caffeine, and aspirin for weakly teratogen; folic acid, isoniazid, and ascorbic acid for none teratogen. The concentration was determined based on the early report. To characterize three germ layer differentiations, we used AFP for endodermal lineage marker, Brachyury for mesodermal lineage marker, and Nestin for ectodermal lineage marker, respectively. One major aspect of the project is the automation of the 3D image acquisition and the accurate quantification of the EB phenotype. For this purpose we have developed a 3D image processing software that analyze the EB morphology and quantify the 3 germs layer differentiation ratio. Quantitative structure-activity relationship (QSAR) analysis is also performed in parallel to increase the robustness of the prediction protocol. In conclusion, we propose a novel way of quantifying and predicting the developmental toxicity in a HCS context using hESC-derived EB, a 3D structure mimicking the early stage of the embryo development. Our approach is highly challenging on many different aspects, but has been already validated on reference compounds.

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Poster Board Number: F-2237

SUSPENSION CULTURE OF HUMAN PLURIPOTENT STEM CELLS IN CONTROLLED, STIRRED BIOREACTORS

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Therapeutic and industrial applications of pluripotent stem cells and their derivatives requires large cell quantities generated in defined conditions. To this end we have translated single cell inoculated suspension cultures of human pluripotent stem cells to stirred tank bioreactors. These systems are widely used in biopharmaceutical industry, allow straight forward scale up and detailed online monitoring of key process parameters. To ensure minimum medium consumption, but in parallel functional integration of all probes mandatory for process monitoring i.e. for pO2 and pH, experiments were performed in 100 ml culture volume in a "mini reactor platform" consisting of 4 independently controlled vessels. By establishing defined parameters for tightly controlled cell inoculation and aggregate formation up to 2 x 108 hPSCs / 100 ml were generated in a single process run in 7 days. Expression of pluripotency markers and cells ability to differentiate into derivates of all three germ layers *in vitro* was maintained, underlining practical utility of this new process. The presented data provide key steps towards scalable mass expansion of human iPS and ES cells thereby enabling translation of stem cell research to (pre)clinical application in relevant large animal models and valuable *in vitro* assays for drug development and validation as well.

Poster Board Number: F-2238

DETECTION AND SEPARATION OF UNDIFFERENTIATED HUMAN PLURIPOTENT STEM CELLS FROM HETEROGENEOUS CELL POPULATIONS USING LECTINS AND NOVEL MONOCLONAL ANTIBODIES

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Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESC) and human induced pluripotent stem cells (hiPSC), have the abilities to indefinitely self-renew and to differentiate into essentially all cell types. While these characteristics make them potential sources of material for a wide range of clinical applications, they also pose potential risks from uncontrolled self-renewal and off-target cellular differentiation. To enhance the reproducibility of *in vitro* differentiation methods, and to decrease the potential for differentiation down off-target paths, it may be desirable to generate homogeneous undifferentiated pluripotent stem cell populations in which all the cells have similar differentiation capacities and responses to differentiation factors. In addition, rapid and dependable methods to detect and remove residual undifferentiated pluripotent cells from differentiated derivatives are essential to ensure the safety of cell populations differentiated from hPSCs for clinical purposes.We have identified lectins specific for pluripotency-associated glycoproteins that can be used for the detection and isolation of viable hPSCs. In addition, we have screened commercially available antibodies for specificity to hPSC cell-surface epitopes and generated novel hPSC-specific monoclonal antibodies and validated them by high-throughput live-cell FACS screening (see related abstract: Carmel O'Brien et al).Using the pluripotency-associated lectin UEA-I in a high-content imaging assay, we have been able to reproducibly detect rare hPSCs in a mixed cell population at a ratio of hPSCs:fibroblasts of 1:100,000. We have also used biotinylated UEA-I in magnetic activated cell separation (MACS) to remove pluripotent cells from mixed cell populations that consist of hPSCs and fibroblasts at a broad range of mixing ratios. The high efficiency of UEA-I-mediated MACS was demonstrated by the quantitative removal of hPSCs from a mixed population of 100 hPSCs and 1 million fibroblasts. We are now testing these methods on populations of neural progenitor cells generated by directed differentiation of hPSCs, as well as on mixed populations consisting of hPSCs spiked into a variety of primary cell types. Since none of the lectins or monoclonal antibodies in our collection is absolutely specific for hPSCs, we anticipate that widely generalizable strategies for detection, enrichment, and removal of hPSCs from mixed populations will involve multiple lectins and antibodies. We are currently optimizing detection and separation methods using several antibodies identified in our screens, including the TG343 antibody (targeting pericellular keratan sulphate proteoglycan antigen) and the novel monoclonal antibody AL27 (targeting the F11R/JAM-A receptor). We have recently coupled the TG343 antibody to a cytotoxic saporin moiety and demonstrated that this reagent results in selective toxicity to pluripotent cells. Our results suggest that the lectins and monoclonal antibodies identified in this study are potentially valuable tools for the quality control of hPSC-derived cell populations destined for clinical use. They can be applied to generate homogeneous populations of undifferentiated hPSCs for use as the starting material for *in vitro* differentiation procedures, and to detect and purge residual undifferentiated pluripotent cells from the resulting differentiated populations.

Poster Board Number: F-2239

GENERATION OF A HUMAN MODEL OF MACHADO-JOSEPH DISEASE BY REPROGRAMMING OF PATIENT FIBROBLASTS

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Machado-Joseph disease is a hereditary neurodegenerative disorder caused by the expansion of a polyglutamine tract in the C-terminus of ataxin-3. The study of the pathogenesis of MJD has been restricted to artificial disease modeling systems such as animal models or cell lines, which present limitations as models of human neurogenetic disorders. With the development of induced pluripotent stem cell (iPS) cell technology disease specific neurons can now be generated from patients' fibroblasts. In the present work we investigated whether dopaminergic neurons can be generated from Machado-Joseph disease patient fibroblasts and provide a model for the study of the disorder in living neurons by reprograming patient fibroblasts into iPS cells followed by differentiation into this particular subtype of neurons. iPS cell lines were generated through the induced expression of the four transcription factors OCT3/4, SOX2, KLF4 and c-MYC using a policistronic SFFV (spleen focus-forming virus promoter)-driven 4-in-1 codon optimized lentiviral vector. Colonies were picked at days 16-22 and mechanically dissociated for replating and passaging. Comprehensive characterization involved alkaline phosphatase activity assay, immunostaining for pluripotency markers (TRA-160, TRA-1-81, SSEA4 and NANOG), qRT-PCR for evaluation of transgene silencing

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and levels and pattern of expression of endogenous pluripotency genes (OCT4, SOX2, NANOG, GDF3, TERT, REX1, ABCG2), and in vitro differentiation studies. The results indicate that the clones are bona fide iPS cells. *In vivo* differentiation studies and neuronal differentiation in dopaminergic neurons from iPS cells are in progress. Functional and electrophysiological studies are also on-going to characterize and compare the molecular state of the induced cell to the target cell, measure its functionality and assess the stability of reprogrammed cell fate of dopaminergic neurons obtained by this approach. It is expected that this new human model of MJD will provide a physiologically-relevant disease model, recapitulating the molecular and cellular phenotypes typical of MJD and opening new opportunities for exploring the disease pathogenesis and for drug screening.

Poster Board Number: F-2240

PURIFICATION OF HUMAN ADIPOSE TISSUE-DERIVED STEM CELLS BY MEMBRANE FILTRATION METHOD

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Adipose-derived stem cells (ADSCs) are a promising cell source in regenerative medicine, of particular utility for cell therapies and tissue engineering, because adipose tissue can easily be harvested in large quantities compared to bone marrow, and ADSCs have high proliferation rates in culture. ADSCs are isolated from adipose tissue by liposuction and centrifugation followed by cultivation on cell culture dishes for at least one passage. The cultivation of cells derived from adipose tissue is necessary to purify ADSCs (i.e., "the culture method" for the purification of ADSCs) because the adipose tissue contains not only ADSCs but also adipose and other types of cells. The culture process for the purification of ADSCs requires several days, at minimum. If ADSCs can be purified from adipose tissue in a short period of time (i.e., less than 2 hrs) by using a cell purification device such as the membrane filtration method, cell therapy and tissue engineering applications using autologous ADSCs might become more efficient. Therefore, we investigated the purification of human ADSCs from a digested solution of adipose tissue by the membrane filtration method in this study, and we compared the purity of ADSCs and the differentiation ability of ADSCs into osteoblasts after purification by the membrane filtration method and the conventional cell culture method. We investigated two filtration methods to purify hADSC, i.e., batch-type and perfusion-type filtration methods. Main differences between these two filtration methods are cell flow direction to the membranes. Polyurethane foaming membranes having 5-12 μm of pore size were used as the membranes for the separation of hADSCs from human adipose tissue. The surface marker of ADSCs (e.g., CD73 and CD90) in the cells in the permeate and recovery solutions were analyzed by flow cytometry whether the mesenchymal stem cells were enriched after permeation through the membranes. The differentiation of cells into osteoblasts, which were separated by the membrane filtration method was evaluated to confirm the enriched hADSC in the permeate solution through the membranes by culture of the cells in induced medium of osteogenic differentiation. We, further, investigated the isolation of ADSCs by the membrane filtration method through surface-modified PU membranes having with various nanosegments (e.g., $-NH_2$, $-SO_3H$, -OH, and -COOH), and compared the isolation efficiency of ADSCs purified through nonmodified PU membranes and surface-modified PU membranes. We found that the cells separated through PU membranes by the perfusion method showed high popuration of ADSCs from surface marker analysis and the highest osteogenic differentiation ability.

Poster Board Number: F-2241

NOVEL CRYOPRESERVATION AGENT FOR EFFECTIVE VITRIFICATION OF HUMAN EMBRYONIC STEM CELLS USING CARBOXYLATED E-POLY-L-LYSINE

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(Purpose) Safe and Stable cryopreservation is one of the most important issues in clinical application of human embryonic stem (hES) cells. DMSO is very popular cryoprotective agent, but its cytotoxity and influence on cell differentiation are not ignored, so the substituted cryoprotectant is desirable. We already reported that a novel cryopreservation reagent with anti-freezing polyampholyte, and it was excellently effective for human iPS cells by vitrification. In this study, we tested that this solution could be applicable to the cryopreservation of hES cells. (Materials & Methods) We already showed the synthesis of the polymeric cryoprotectant, carboxylated ε-poly-L-lysine (COOH-PLL) [1. Matsumura K, et al. Polyampholytes as low toxic efficient cryoprotective agents with antifreeze protein properties. Biomaterials 30: 4842-4849 (2009), 2. Matsumura K, et al. Effective vitrification of human induced pluripotent stem cells using carboxylated ε-poly-L-lysine. Cryobiology, 63, 76-83 (2011)]. hES cells that we used were KhES1 and KhES3 supplied from Kyoto University. The hES cells at the condition of colonies were cryopreserved by vitrification with our cryopreservation agent (StemCell Keep ®; Bio-Verde, Kyoto, Japan) containing 10% carboxylated poly-L-lysine, 6.5 M ethylene glycol and 0.75M sucrose. After freezing in liquid nitrogen, hES cells were thawed again, and cultured on SNL feeder layer, and observed their morphology. We checked their undifferential abilities by alkaline-phosphates staining and immunohisochemical staining for stem cell markers, OCT-3/4, SSEA-4 and TRA-1-60, and also their karyotype analysis was performed. (Results & Discussion) When hES cells were cryopreserved with our new solution (StemCell Keep ®) containing anti-freezing polyampholyte, it was shown that the cell growth was higher than that with the existing DAP213 solution containing 2M DMSO, 1 M acetamide and 3 M propylene glycol, and the total cell number of the former was about twice fold as high as that of the latter. The hES cells, which were frozen with StemCell Keep ®, were thawed and cultured in single cell suspension on Matrigel-coated dish, these cells could be easily attached to the bottom of the dish on the next day, but fewer cells frozen with DAP213 were attached there. The alkaline phosphates staining and the immunostaining clearly showed the undifferential colonies in the culture of hES cells frozen with our solution. The karyotype analysis of those cells also showed normal chromosome components. Our anti-freezing polyampholyte (StemCell Keep ®) become effectively a good reagent for hES-cell cryopreservation in stead of DAP213. Furthermore, slow freezing is better cryopreservation of hES cells for clinical application in future, so we try to make a new cryoprotective agent for slow freezing.

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Poster Board Number: F-2242

HUMAN EMBRYONIC STEM CELL (HESC) LINES; MODELS FOR CHROMOSOMAL AND GENETIC DISEASES

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hESC research is performed with IVF embryos derived from three major sources. These include donated normal discarded embryos, chromosomally abnormal embryos (monosomy, trisomy and mosaics) which would otherwise be discarded after preimplantation genetic diagnosis or screening (PGD or PGS), and embryos carrying specific genetic diseases. Stem cells derived from the latter two sources can represent excellent in vitro models to study specific chromosomal and genetic diseases. hESC lines were derived from blastocysts obtained after PGS or PGD analysis on day 3 of embryo development. Blastomeres were screened for chromosomal abnormalities by FISH or tested for the specific gene mutations or linked markers. hESC lines were fully characterized and tested for pluripotency in vitro and *in vivo*. Out of 39 hESC lines: 4 lines were derived from donated IVF embryos, 12 from PGS aneuploid diagnosed embryos, 5 with unbalanced chromosomal translocation and 18 from embryos with single gene disorders. Derivation efficiency varied between the groups: hESC derivation efficiency was 30% efficiency for IVF and PGD embryos whereas chromosomally abnormal embryos exhibited a lower derivation efficiency at 15%. Overall, derivation efficiency depended on the presence of the inner cell mass (ICM) in the blastocyst, the introduction of laser ICM isolation and utilizing a novel hESC-conditioned medium. With the latter innovations, a 50% hESC derivation rate can be achieved. In the PGS group, 9/12 lines were found to have a normal chromosomal complement by karyotypic and SNP analysis; 3 were abnormal with only one showing the corresponding abnormality exhibited by its progenitor day 3 embryo. These results can be explained by the inefficiency of a 9 chromosome FISH analysis on day 3 embryos, by blastomeric mosaicism on day 3 embryos or may be a result of cell selection at the hESC level (i.e. preferential growth and dominance of euploid over aneuploid cells). No uniparental disomy (UPD) was observed by microsatellite and SNP analysis in these lines. Extended in vitro cultures of hESC promotes the acquisition of oncogenic genomic changes observed by SNP analysis resulting in chromosomal aneuploidy (trisomy observed in 3 lines). In the PGD group, 3 hESC lines were derived from fragile X affected embryos. Fragile X analysis (Southern blot) of the hESC lines showed one normal line (30 CGG repeats); one permutated (73 repeats) and one fully mutated line (>200 repeats). The list of other affected hESC lines include: BRCA1 and BRCA2, Beta-Thalassemia, Huntington, Cystic Fibrosis, Retinoblastoma, Hemophilia A, Achondroplasia, and Dystonia. hESC lines from affected embryos represent a potential in vitro model for unraveling developmentally-regulated mechanisms associated with chromosomal and genetic abnormalities. PGS abnormal embryos can serve as an alternative source for normal euploid lines.

Poster Board Number: F-2243

A NOVEL METHOD FOR HIGH-THROUGHPUT KARYOTYPING OF HUMAN PLURIPOTENT CELLS

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Genomic integrity of human pluripotent stem cell (hPSC) lines requires routine monitoring, since *in vitro* maintenance of stem cells is associated with the accumulation of karyotypic abnormalities. We have tested a novel karyotyping assay, which enables frequent monitoring of the genomic integrity of pluripotent cells in cost-efficient and high-throughput manner. The assay measures DNA copy numbers at the chromosome arm resolution utilizing bacterial artificial chromosome (BAC) probes immobilized onto color-encoded polystyrene microspheres distinguishable by a luminometer. We have compared the novel method to conventional G-banding and array based methods, and the data obtained with bead based system is in good concordance with the other analyses. The method provides a fast and easy tool for detection of chromosomal abnormalities, and enables routine screening of stem cell lines in a cost-efficient high-throughput manner.

Poster Board Number: F-2244

A DE-NOVO DESIGNED CXCR4 SYNTHETIC AGONIST DIRECTS TARGETED HUMAN NEURAL STEM CELL MIGRATION

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Human neural stem cells (hNSCs) respond to many spatial and temporal signals along with their integration into the central nervous system (CNS). Chemokines are among these transiently expressed signals, often supplied by microglia and invading macrophages at "niches of injury" within these areas. The precise engagement of neural progenitors was demonstrated by the significant elevation of stromal cell derived factor 1α (SDF-1α). Activation of CXCR4, the cognate receptor of SDF-1α, triggers a series of intracellular molecular programs mediating multiple reparative actions, including the trafficking and survival of hNSCs. Based on these existing contexts, a major challenge for the success of CNS regeneration is to create an "optimized" environment for highly effective stem cell engagement. Here we show a de-novo designed synthetic peptide being highly potent in mobilizing hNSCs both *in vitro* and inside the engrafted healthy adult mouse brains. Cell-based experiments and molecular modeling have demonstrated the agonist peptide's capacity in CXCR4-selective binding and signaling. Further in-vivo investigations supported its effectiveness in guidance of hNSC engraftment without associating significant microglia activities like natural SDF-1α. Our results thus validated a novel design strategy for "bi-functional" CXCR4 agonists based on concurrent structural understandings to chemokine ligand-receptor interactions. We anticipate these explorations will promote beneficial applications of chemokine analogs into the cutting-edge stem cell therapeutics. Since the detailed structural information of seven-transmembrane G-protein coupled receptors (GPCRs) remains largely unknown, these results may also advance the fields of drug development that densely target to this superfamily for perturbations of sizable human diseases.

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Poster Board Number: F-2245

THE IMPORTANCE OF USING SMALL-SCALE BIOREACTOR MIMICS TO SCALE-UP HUMAN EMBRYONIC STEM CELL CULTURE

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The commercialisation of allogeneic cell therapies for large patient populations is reliant on both minimising manual handling and realising economies of scale. Thus the use of single use large-scale bioreactors that are specifically designed to replace the passaging and expansion of adherent cell cultures will be required. The Integrity Xpansion multiplate bioreactor from ATMI has an integrated low shear stress discontinuous mixing system that is operated to control the growth environment to user-defined optimum values of pH and dissolved oxygen. The largest size of this bioreactor has a growth area of 11.m2 (equivalent to 18 multitray 10 layer stacks) and a working volume of 19.8L with external dimensions of 35x60cm - minimising the space needed in expensive processing facilities compared to existing technologies. However experimentation to optimise a process on such a scale is prohibitively expensive and wastes resources. Therefore before any large scale experimentation human embryonic stem cells were grown on the Xpansion One system; a 128cm2 plate which mimics the fluid dynamics and gas exchange control system exhibited on the far larger 0.6m^2, 3.m^2 and 11.m^2 Integrity Xpansion bioreactors. Data will be presented from an initial feasibility study that attempted to optimise cell growth whilst maintaining pluripotency through the exploration of the control mechanism on the Xpansion One plate. Variables such as flow rate across the plate, pH set points and oxygen/CO2 composition were investigated to try and improve performance in relation to T25 flask controls. Metrics such as glucose, lactate, cell growth, viability, pH, dissolved oxygen and pluripotency marker expression via flow cytometry and qPCR were tracked and measured to relate respective performances. The data presented will highlight the significant impact of what would appear to be relatively small deviations in environmental conditions, such as pH, has on cell growth. This makes environmental control systems and use of small-scale mimics of critical consideration when moving cell culture away from traditional methods to large-scale novel bioreactors for commercial production.

Poster Board Number: F-2246

A ROTENONE SUSCEPTIBILITY PHENOTYPE IN OLFACTORY NEUROSPHERE-DERIVED CELLS FROM IDIOPATHIC PARKINSON'S DISEASE PATIENTS

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One powerful, but as yet mostly untapped, use of stem / progenitor cells is in the identification of mechanisms underlying complex diseases, such as idiopathic Parkinson's disease (iPD). We have developed an in vitro system using human olfactory neurosphere-derived (hONS) cell lines to investigate iPD. hONS cells are homogenous populations of cells expressing characteristics of ecto-mesenchymal progenitor cells. We have previously detected significant deficiencies in glutathione (p=0.016) and MTS metabolism (p=0.019) in 26 iPD hONS cell lines compared with 28 control hONS. In addition these functional differences in iPD hONS can be restored to normal levels by activation of the Nrf2 anti-oxidant stress response pathway. These results raised the possibility that iPD hONS cells might be more sensitive to cellular stresses and hence undergo apoptosis more readily, as has been suggested for dopaminergic neurons in iPD patients *in vivo*. To test hONS cells as a model of iPD, we subjected them to a range of cellular stresses including; mitochondrial complex I (using rotenone), proteasomal (epoxomycin), oxidative (H2O2), endoplasmic reticulum (tunicamycin), DNA damage (camptothecin) and lysosomal (chloroquine). We found hONS cells from iPD patients apoptosed more readily than control hONS cells (8 vs 8 $p < 0.004$) following a single exposure to 50nM rotenone (mitochondrial complex I inhibitor). A similar, though somewhat lesser sensitivity was detected in iPD hONS cells following exposure to hydrogen peroxide (oxidative stress). There was no disease-specific sensitivity following exposure to the other stressors. To validate these data we exposed a total of 19 iPD and 20 Control hONS cell lines to rotenone, over multiple biological replicates and confirmed that hONS cells from iPD patients display a disease-specific susceptibility phenotype to rotenone (p=2.62E-20). Exposure of hONS cells to another mitochondrial complex I inhibitor (Piericidin A) produced a similar susceptibility phenotype (p=7.9E-9). To examine where in the cell, upstream or downstream of the mitochondria, the sensitivity lay we measured complex I activity in untreated hONS cells from iPD (14 lines) and healthy Controls (12 lines). We detected 25% less CI activity in iPD hONS in untreated cells ($p<0.0001$). However, we also observed that CI activity was not directly correlated with rotenone susceptibility in individual hONS cell lines. Therefore hONS cells are a viable cell system with which to investigate the mechanisms underlying iPD. hONS cells display reproducible, quantifiable differences in cell function which correlate with disease status, but not other variables (age of donor, passage number, gender, age of disease onset, medication regime, etc). In addition, as the assays are amenable to high throughput screening, we are currently using iPD hONS cells to identify compounds which modulate rotenone susceptibility.

Poster Board Number: F-2247

SYSTEMS BIOLOGY APPROACH TO STUDY STEM AND PROGENITOR CELLS OF NORMAL AND MALIGNANT HUMAN TISSUES.

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Many, if not all organs and tissues consist of self-renewing stem cells that give rise to distinct, sequential progenitors with increasingly limited development potential, ultimately producing functional mature cells. All malignancies develop from cells within such hierarchies, requiring progression of events resulting in tumor cells that are capable of self-renewal, survival, migration, and likely also differentiation. The identification and characterization of stem, progenitor, and mature cells within normal and diseased tissue are not only critical for the understanding of underlying biology but also in developing more effective therapeutic strategies. Previous attempts to identify markers for cells at hierarchical stages of tissue

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differentiation involved either 1) large screening studies using antibody libraries or gene expression arrays, or 2) focused trials of established markers identified in other normal and diseased tissues. Unfortunately, this "random" approach is insufficient to trace complex cellular differentiation stages, and thus most often fails. Therefore a systematic approach to identify cells within tissue differentiation hierarchies is required. We applied systematic computational approaches to identify markers of stem and progenitor cells by analyzing publicly available, high-throughput gene expression datasets consisting of more than 2 billion measurement points, and subsequently to validate them using tissue microarrays. We used a new method called MiDReG (Mining Developmentally Regulated Genes) that uses Boolean implications to successfully predict genes in developmental pathways. We developed a new software tool called HEGEMON (Hierarchical Exploration of Gene Expression Microarray Online) to identify genes expressed in the stem and progenitor cells in malignant tissue development. HEGEMON explores gene expression data with its clinical information using a scatterplot of gene expression values from two genes and provides a simple framework for automatic selection of genes correlated with distinct patient information, e.g. progression and survival. Using the above tools we demonstrate a new concept that human cancers can be used as a platform to study normal developmental steps of the human tissues. We use examples of human bladder and colon cancer to show the power of this computational approach.

Poster Board Number: F-2248

VIALS FOR CLINICAL DEVELOPMENT OF CELL THERAPY PRODUCTS: TEST OF VIABILITY AND FUNCTIONALITY OF MSCS IN VIALS AFTER STORAGE AND TRANSPORT ON COLD AND CRYOGENIC CONDITIONS

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Many current cell-based products are produced, frozen, stored and delivered to the clinical site in intravenous (IV) bags or polypropylene screw-cap bottles. However, these containers may not be suitable for companies seeking to supply these products for clinical treatment on a commercial scale. As the industry expands and moves to commercialize cell-based therapies, there is a clear need for a scalable, vial-based packaging system conducive for pharmaceutical fill finish operations. The vial system should be suitable to package, store and transport cell therapy products at cold or cryogenic temperatures, and meet pharmaceutical compendial requirements. This study investigated the suitability of Daikyo Crystal Zenith (CZ) plastic vials, made of cyclic olefin polymer, to contain, store and transport biopharmaceutical preparations at these low temperatures. CZ vials (0.5, 5.0 and 30mL capacity) with several closure systems were filled with cells from a mesenchymal stem cell (MSC) line and stored at either -85°C or -196°C for six months. Vials were tested for (a) durability and integrity of the filled vial utilizing a 1-meter drop test, and (b) ability to maintain viability and functionality of cryopreserved cells. The results showed that there was no evidence of external damage on vial surfaces and no cracks or damage were seen on closure systems. Dye immersion studies using spectrophotometer measurements indicated container durability (break-resistance) with no failures. Temperature and time of storage had no effect on the durability (break-resistance) of vials after a drop test. Post-thaw viability utilizing a dye exclusion assay was >95% in all samples. Stored cells exhibited rapid recovery 2 hours post-thaw and cultures were ~70% confluent within 5-7 days, consistent with non-frozen controls and indicative of functional recovery. Doubling times were consistent over all vial sizes. Doubling rates for cells stored in CZ vials were 1.84±0.68 days (1 month), and 1.79±0.71 (6 months) compared to frozen controls 1.99±0.49 days (1 month) and 1.68±0.85 (6 months) and compared to fresh controls. No statistically significant difference ($p>0.05$) was observed. Vials were durable and allowed for preservation and maintenance of cell viability and functionality. CZ vials are 100% vision inspected, have a low extractables profile, and lower permeability of gas and moisture compared to polypropylene. Appropriate Drug Master Files and technical dossier are filed or available per regional requirements. The CZ vial container system is suitable for low-temperature frozen and cryopreserved storage of cell products.

Poster Board Number: F-2249

STUDYING METABOLIC STATUS OF HUMAN PLURIPOTENT STEM CELLS BY OPTIMIZED BIOCHEMICAL ASSAYS

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Nicotinamide adenine dinucleotide (NAD) and its related pathways have been suggested as important regulators of cellular physiology. Thus, in addition to being used as a co-factor and a redox carrier in key stages of both cytoplasmic glycolysis and mitochondrial oxidative phosphorylation, NAD serves as a substrate for acetylation and ADP-ribosylation of many regulatory proteins. In human embryonic stem cells (hESCs) it is recognized that they obtain their energy through aerobic glycolysis rather than via oxidative phosphorylation. However, very little is known about NAD homeostasis in hESCs. Therefore we aimed to study whether intracellular metabolic parameters correlate with changes of stem cell fate, and, in particular, how metabolic parameters are affected by different culture conditions, how they relate to the state of stemness and what are the metabolic parameters that correlate with the onset of differentiation. We first set out to establish reliable, sensitive, reproducible and affordable biochemical assays for important metabolites (NAD+, NADH, ATP, lactate etc) and optimized them for applications in hESCs. For optimizations we employed DynaFit (BioKin, Ltd) software for fitting the cycling reaction velocities to the concentrations of metabolites. In addition, we developed a model of hESCs with a compromised metabolism using FK866, an inhibitor of nicotinamide phosphoribosyltransferase, which is a key enzyme in a NAD rescue pathway. We found that NAD+/NADH ratio is relatively low in undifferentiated hESCs compared with differentiated cells. This is in line with a glycolytic nature of the stem cells, in which NAD+ is consumed while NADH is accumulated. Significantly, this ratio rapidly increases in hESCs that are supplied with fresh medium. We speculate that the pool of NADH is being quickly utilized for reduction of pyruvate to lactate followed by its release into the medium. We currently investigate why hESCs invest such a substantial amount of energy in form of NADH to produce lactate. Is it solely due to a hypoxic nature of the stem cells, or can it have any meaningful regulatory significance? We hypothesize that the production of lactate is used in hESCs for re-oxidation of NAD in order to support continuous glycolysis. In addition, it will be interesting to investigate whether such rapid changes of NAD+/NADH ratio caused by change of media correlate with known phenomenon of oscillation in the expression of marker stem cell genes. In summary, we developed set of sensitive and robust generic biochemical assays for studying metabolic parameters in hESCs. Our data might

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contribute to the development of more reliable culture conditions for undifferentiated hESCs and, perhaps, to the development of more efficient differentiation protocols.

Poster Board Number: F-2250

MULTIMODAL IMAGING OF ALLOGENEIC IPS CELL-BASED CARDIAC RESYNCHRONIZATION THERAPY IN MOUSE ISCHEMIC HEART FAILURE MODEL

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OBJECTIVES Induced pluripotent stem cells (iPS) repair myocardial injury in the setting of acute myocardial infarction. However, the impact of iPS therapy on regional electromechanical disturbance and its contribution to global cardiac function/structure has not been tested. This study tests the long-term impact of stem cell-based intervention on infarction-provoked cardiac electro- and mechanical-dyssynchrony through high-resolution imaging in a murine, chronic ischemic heart failure model. BACKGROUND Heart failure is the leading cause of morbidity and mortality worldwide. Cardiac dyssynchrony, from which up to 60% of heart failure patients suffer, is serious consequence of myocardial infarction that aggravates disease outcome. As current modalities are merely palliative, strategies that would ensure tissue repair and synchronize dysfunctional myocardium are thus warranted. METHODS Mouse embryonic fibroblasts were transduced with stemness factors OCT3/4, SOX2, KLF4, and c-MYC packaged in lentivirus to produce iPS clones which meet criteria of pluripotent stringency. Cells were labeled with LacZ or luciferase. Male, 8-12 week olds mice (n=42) underwent permanent ligation of the left coronary artery. In a blinded fashion, infarcted mice were randomized into 3 groups, no treatment, fibroblast- and iPS-treated groups (epicardial delivery of 200,000 cells/heart into mapped infarcted areas) within 30 min post-ligation, and followed by M-mode/2-D/3-D/speckle tracking echocardiography (30-MHz), electrophysiology, *in vivo* imaging, and ultimately pathology, up to 3 months post-infarction. In speckle tracking, left ventricular endocardium was mapped with a previously unachievable spatial and temporal resolution (48 sampling points with 70-400 μm intervals at >200 frame/s). Myocardial electro-mechanical dynamics were deconvoluted in a multiparametric fashion resolving peak systolic strain/velocity, and time-to-peak systolic strain/velocity. Patterns of abnormal ventricular contractility were collectively diagnosed based on degree, timing, and direction of myocardial motions. RESULTS Compared with untreated infarction, regional iPS intervention, but not delivery of parental fibroblasts, restored myocardial contractility in targeted infarcted foci, and nullified conduction delay in adjacent non-infarcted regions. Local iPS therapy prevented or normalized abnormal motion patterns correcting decrease in peak systolic strain/velocity, disparity of time-to-peak systolic strain/velocity, and pathological systolic stretch. These benefits on regional myocardial dynamics were detected within 10 days post iPS implantation. Cell engraftment was documented by *in vivo* imaging. Histological assessment demonstrated remuscularization with reduced interstitial fibrosis in iPS treated ventricles. Focal benefit of iPS cell intervention translated globally into improved left ventricular ejection fraction, reversal of electrical and structural remodeling, and protection against overt decompensation. No adverse effects were detected throughout the 3 months follow-up. CONCLUSIONS Thus, targeted iPS cell transplantation synchronized infarcted regions eliminating inhomogeneous contraction and conduction disparity in cardiomyopathic ventricles. By enabling focal repair, stem cell therapy offers a prophylactic resynchronization strategy to avert organ failure post-infarction.

Poster Board Number: F-2251

EXPANSION OF UNDIFFERENTIATED MOUSE EMBRYONIC STEM CELLS IN SUSPENSION BIOREACTOR ON VARIOUS SHEAR CONDITIONS

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Objectives: Embryonic stem cell (ES cell) and induced pluripotent stem cell (iPS cell) have an almost unlimited capacity for self-renewal and potential to differentiate into multiple cell lineages. These cells have much attention as a cell source for a regenerative medicine technology. To realize the therapeutic potential of these stem cells, it is necessary to establish an effective differentiation method from undifferentiated stem cell to specific matured cell. In parallel, a mass cultivation system for undifferentiated cells must be established to use stem cell near future. Two-dimensional monolayer culture as a traditional culture system is difficult to scale-up. We focus on stirred-tank as a culture vessel for ES cell culture. In this study, we evaluate the effect of shear condition on ES cells proliferation in suspension bioreactor. Materials and Methods: Mouse ES cells (129 Line) were grown on a feeder layer. In the suspension culture system, a single-cell suspension of 5.0×10^5 cells was added to a 100 ml spinner vessel containing 50 ml of culture medium supplemented with 1000 U/ml LIF. The culture was stirred continuously for 3 days at various agitation rate; from 40 to 250 rpm. Then, cells were harvested and were digested by the enzymatic dissociation process to obtain single-cell suspension. The cells were enumerated and reseeded back in the spinner vessel. Every 3 days, ES cells were passaged in a same procedure. Results: ES cells formed multicellular aggregates spontaneously because of highly E-cadherin expression. The mean diameter of aggregates was increased with culture time. The average aggregate diameter remained less than 250 micrometers for 3 days of culture. In low shear condition, ES cells did not proliferate because of transport limitation of oxygen or nutrients. The fold expansion increased with increasing shear stress, then expansion ratio decreased because of excessive shear stress. It was found that high cell density culture could be achieved in the suspension bioreactors as long as the maximum shear stress was maintained within the range of 0.4 and 0.6 Pa. The resulting bioprocess achieved a 11-fold expansion in 3 days. The high expansion ratio was maintained at least 5 serial passaging. The pH was maintained above 7.1. ES cell aggregates were harvested and were evaluated its pluripotent capacity. Pluripotency markers of Oct-4 and SSEA-1 were evaluated using flow cytometry analysis and gene expression profiles. Oct-4 mRNA level was maintained at a level of undifferentiated state. SSEA-1 expression was above 80% throughout the entire suspension culture period. These results showed that the ES cells within the aggregates maintained in an undifferentiated state throughout the culture period. Upon removal of LIF, ES cell aggregates showed the capability to differentiate into cells derived from all three embryonic germ layers. Conclusion: ES cells showed sustained proliferation in a controlled suspension bioreactor. Their undifferentiated state and pluripotency were maintained throughout the culture period. These results indicated that this suspension culture process provides an alternative to the conventional culture process on feeder cells.

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Poster Board Number: F-2252

CHARACTERIZATION OF INDUCTION OF DIABETES IN JUVENILE CYNOMOLGUS MONKEYS WITH DIFFERENT DOSES OF STREPTOZOTOCIN

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The juvenile (2-3 years old) cynomolgus monkeys were frequently used as recipients in nonhuman primate islet transplantation studies. To examine the effects of different doses of streptozotocin (STZ) and find an optimal dose to induce diabetes in those monkeys, fifteen juvenile (2-3 years old) cynomolgus monkeys were separated into three groups and administered with different doses of STZ (100mg/kg, 68mg/kg or 60mg/kg). Basal and glucose-stimulated blood glucose, insulin, C-peptide levels and body weight were monitored. Tests of liver and kidney functions and immunohistochemistry of pancreata were performed before and after STZ treatment. The results of this study showed that 100mg/kg and 68mg/kg of STZ-treated monkeys exhibited continuous hyperglycemia caused by nearly complete loss of islet β cells. Two monkeys received 60mg/kg of STZ but only one became completely diabetic. During the first week following STZ injection, liver and renal function test values only slightly increased in the three groups of monkeys. However, serum total bile acid level was significantly increased in monkeys treated with 100mg/kg than those treated with 68mg/kg of STZ 24 hours post-STZ ($p<0.05$). These data suggest that 100mg/kg and 68mg/kg of STZ both can safely induce diabetes in cynomolgus monkeys aged 2-3 years, but 68mg/kg of STZ, rather than 100mg/kg of STZ, may be more appropriate for inducing diabetes in these monkeys. Furthermore, body surface area instead of body weight was a more reliable determinant of dosage; 700mg/m^2 of STZ should be the lower limit for diabetes induction in juvenile monkeys.

Poster Board Number: F-2253

BUILDING BLOCKS FOR A BETTER UNDERSTANDING OF MOUSE MESENCHYMAL STEM CELL-ECM PROTEIN INTERACTION

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Growing cells are surrounded by a mixture of soluble molecules and insoluble carbohydrates, lipids, mineral surface and proteins. Many different proteins along with glycosaminoglycans and neighbouring cells combine to create a complex and dynamic network in which cells integrate and interact. Over the past 20 year's site-directed mutagenesis methods have allowed us to engineer proteins; inserting, deleting and replacing amino acids. Engineered proteins are now being used in medical research to improve treatments of human disease and in the industry. *In vivo* proteins and other ECM components form an interlinking mesh. A way to mimic this natural architecture of ECM is through crosslinking artificial polymers. Ultimately, one could imagine an ideal 3-D cell culture system fabricated from a synthetic biological material with defined constituents and which allows the exploration of specific interactions between different cell lines and the surrounding microenvironment. Furthermore, using scaffolds made from proteins might allow us to create a more realistic microenvironment for cells, not only because they are nanoscale fibers with nanoscale pores allowing access to oxygen, hormones and nutrients and removal of waste products but also due to their biocompatibility and biodegradability. We report here flexible protein nanofibers, up to 1.5 µm long which can be crosslinked with a non-toxic and non-immunogenic chemical compound - PEG, forming pores smaller than 100 nm. This protein hydrogel is composed of interchangeable folding units which can be used to incorporate cell interacting peptide motifs. It is robust and, in the unmodified state highly protease resistant. The controlled engineering of sites within the polymer allows us to study their implication in cell attachment, survival and proliferation. In this study we used different mammalian cell lines and mouse mesenchymal stem cells to understand how cells behave in the presence of this biomaterial, with particular focus on the formation of focal adhesion structures, changes in cytoskeletal rearrangement and nuclear and cell morphology. Our preliminary results have shown a possible cell growth inhibition when cells are in contact with the hydrogel scaffold without any motif associated. In contrast, the incorporation of a commonly used motif derived from fibronectin - RGDS, can invert the scenario of cell growth; promoting cell attachment, survival and proliferation. Future studies will elucidate the versatility and potential for this peptide hydrogel to influence human mesenchymal stem cell differentiation. This work was supported by MRC Industrial Collaborative Studentship.

Poster Board Number: F-2254

A VERSATILE TRANSGENIC SYSTEM IN COMMON MARMOSET EMBRYONIC STEM CELLS USING RECOMBINASE-MEDIATED CASSETTE EXCHANGE

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Genetic modification is an essential technique for maximizing the potential of non-human primate ES cells. The common marmoset (Callithrix jacchus), a non-human primate, is expected to be a useful transgenic model for preclinical studies. However, genetic modification in common marmoset ES (cmES) cells has not yet been adequately developed. Recombinase-mediated cassette exchange (RMCE), a transgenic strategy using recombinase activity, enables site-specific integration of a transgene efficiently by inserting a cassette flanked by a pair of heterospecific lox sites into a genomic locus in advance. This method is highly advantageous relative to conventional transgenesis using non-homologous end joining because of its predictable, reproducible and stable expression from a single copy transgene. To establish this site-specific transgenic system using RMCE in cmES cells, we inserted the EGFP gene with heterotypic lox sites into the [[Unsupported Character - Symbol Font ]]-actin locus of the cmES cells by gene targeting. The resulting knock-in ES cell line, BR29, expressed EGFP stably under the control of the endogenous [[Unsupported Character - Symbol Font ]]-actin promoter. Using inserted heterotypic lox sites, we have established various transgenic cmES cell lines by RMCE. To name a few: We have established herpes simplex virus-thymidine kinase (HSV-tk) transgenic cmES cell line. These cell line showed sensitivity against ganciclovir (GCV). The growth of tumor cells originating from the cell line was significantly suppressed by the administration of GCV. We also established tetracycline-transactivator (tTA) and reverse tTA (rtTA) transgenic ES cell lines to build doxycycline (DOX) inducible expression system. The expression of transgenes, which have tetracycline response element promoter,

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could be regulated by addition of DOX in these cell lines. Furthermore, transgenic cmES cell lines harboring pluripotency-related transcription factors, such as NANOG, KLF2, KLF4 and KLF5, have been established by using RMCE. These transgenic cmES cell lines showed stable expression of each transgenes over several passages. These results indicate that this transgenic strategy is expected to be useful for preclinical studies and stem cell biology.

Poster Board Number: F-2255

A SINGLE CELL AND FEEDER FREE CULTURE SYSTEM FOR MONKEY EMBRYONIC STEM CELLS.

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Human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) hold great promise for regenerative medicine and drug discovery applications. Primate pluripotent stem cells (PSCs) including human ESCs and iPSCs are conventionally grown on fibroblast feeders in culture medium supplemented with fetal bovine serum or a serum replacement, however, these components are not compatible with clinical applications and desired differentiation. In addition, PSCs undergo massive apoptosis after a complete cell dissociation. Therefore they hardly adapt to a single cell culture condition, which is useful for clonal isolation following genetic engineering, large-scale propagation and cryopreservation. The simple and efficient culture method is required for the practical uses. Here we demonstrate a successful culture system using a serum-free medium supplemented with FGF and Activin, which permits single-cell passage and feeder-free maintenance of primate PSCs. In this system, the cynomolgus monkey ESCs, CMK6 cell line, have been maintained under feeder-free condition and propagated with a high proliferation rate as monolayer cells, without colony formation, that have undifferentiated cell morphology with a high nucleus to cytoplasm ratio. They can efficiently grow beyond 40 passages by single cell dissociation with trypsin treatment. Immunocytochemical analyses show PSCs have a characteristic expression pattern of typical pluripotency markers such as Nanog, Oct-4, Sox2 as well as that of cell surface markers including SSEA-4, TRA-1-60 and TRA-1-81, indicating their undifferentiated and pluripotent state. And they have kept the alkaline phosphatase activity and normal karyotype. And we then confirmed that the culture system allows for effective cryopreservation of single dissociated cells and efficient clonal isolation after gene transfer. Thus, the culture system described here enables more efficient and practical usage of primate PSCs for the clinical and research applications. In this report, we will also present our study in which neural cells could be generated effectively from monkey ESCs to examine their *in vitro* differentiation ability.

Poster Board Number: F-2256

PRODUCTION OF EMBRYONIC STEM CELLS DERIVED FROM SOMATIC CELL NUCLEAR TRANSFER EMBRYOS RECONSTRUCTED FROM OOCYTES PRODUCEDBY *IN VITRO* OVARIAN FOLLICLE CULTURE AND FOLLOWING *IN VITRO* MATURATION IN RABBIT

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In spite of the recent advances in research for patient-specific cell transplantation therapy using induced pluripotent stem cells (iPSCs), the study of embryonic stem cells (ESCs) is still important, because ESCs are derived from embryos and possess normal karyotypes. Furthermore, iPSCs could contain some non-negligible differences between normal ESCs for gene expression profiles, DNA methylation statuses or differentiation potentials. Therefore, notably reprogramming of somatic cells in oocyte cytoplasm and generation of the ESCs via somatic cell-nuclear transplantation (SCNT) may continue to be an important approach to elucidate mechanisms of reprogramming and the nature of pluripotent stem cells in mammals. Unfortunately, progression towards these aims has moved slowly due to legal, ethical and social considerations limiting the availability of oocytes. Many immature follicles remain in the ovarian cortex after ovulation of the dominant follicles in both natural and artificial menstrual cycles. Therefore if we can utilize the oocyte in the immature ovarian follicles, we can gain an important source of oocytes for stem cell research. Here, we reported the successful production of SCNT-ESCs from oocytes produced from immature follicles at early-antral stage that remained after superovulation of rabbits. We collected the immature follicles from ovaries of FSH and hCG treated female rabbits, cultured them for 7 days *in vitro*, induced maturation and obtained the full-size oocytes. We then enucleated them, and reconstructed the SCNT embryos with the oocytes and GFP-expressing rabbit fibroblasts. A total of 121 oocytes were reconstructed, and of these 30 embryos developed to the blastocyst stage. By further culture of the inner cell masses on MEF feeder cells in ESC medium containing bFGF, we established 5 final ES cell lines. The rabbit SCNT-ESCs produced from oocytes grown and matured *in vitro* (referred as IVG-NT-ESCs) showed morphology typical of the primed-pluripotent stem cells like human ESCs, expressed pluripotent markers such as Nanog and POU5f1, and displayed multiple differentiation potentials. These results show the possibility that immature follicle remaining in the ovary after superovulation can be an important source for stem cell production or for research materials used in reprogramming studies.

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Poster Board Number: F-2257

THE APPLICATION OF NANOTECHNOLOGY WITH MESENCHYMAL STEM CELL CULTURE TO DEVELOP A SKIN SUBSTITUTE FOR BURN PATIENTS

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The association of stem cells (SCs) with biomaterials promises to be the protagonist for future regenerative medicine in the treatment of tissue and organ lesions. Available treatment, especially in large burns and chronic wounds, is insufficient to prevent scarring and promote healing of the patient. Because of this, the skin regeneration is an important area for tissue engineering (TE). Aiming at the utilization of the scaffolds of poly-D,L-lactic acid (PDLLA) associated or not with *Spirulina* biomass (PDLLA/*Sp*) (patent applied for) in skin wounds, mesenchymal stem cells (MSCs) from kidney of mice, were seeded onto nanofibres produced by electrospinning (ES). The antinflamatory and antimicrobian effects of the microalga, *Spirulina*, are interesting for skin application. The biodegradable and biocompatible matrices produced were evaluated for morphology, fibre diameter and pore size by scanning electron microscopy. Biological tests were performed as follows: (1) cell adhesion after 6 h of incubation using DAPI staining, (2) cell viability after 1, 4, 7 and 14 days cultivation by MTT assay and (3) cytotoxicity assay on days 4, 7 and 14, through the dosage of the enzyme lactate dehydrogenase (LDH). The molds for growing MSCs were implanted in mice with skin defects that mimic burns. Three groups were tested: (1) PDLLA and PDLLA/*Sp* scaffolds with MSCs; (2) the same scaffolds without MSCs; and (3) animals injured without scaffolds. For groups 1 and 2, a protective cover of 100 nm thick PDLLA scaffolds was used. The MSCs were characterized by immunophenotyping profile using flow cytometry and differentiated into chondroblasts, osteoblasts and adipocytes. The fibre diameter and pore size of the scaffolds obtained for PDLLA were 276±65.9 nm and 2,569±1,279 µm and for PDLLA/Sp 263±82 nm and 2,395±1,047 µm, respectively. The adhesion assay showed that the cells adhere more on PDLLA/Sp scaffolds than only PDLLA. There is a statistical difference between these two groups but both are similar to the control group (cells cultivated directly on the well). In relation to the viability assay, in three points of measurement - days 1, 4 and 14 - there was no statistical difference between the groups. On day 7 the number of live cells on the PDLLA scaffolds was statistically lower than the control group. On the other hand, no statistical difference was observed in the number of viable cells on the PDLLA/*Sp* compared with the control and PDLLA groups. Both scaffolds were atoxic for the SCs, presenting a dosage of LDH much lower than the Triton group, which represents the maximum toxicity. In respect to the animal experiments, the scaffolds implanted in animals tolerated the mechanical stress up to two weeks without breaking. In group 3, the wound seemed to be more disorganized and with a larger bloody area when compared to the other groups. The preliminary results from immunohistochemistry analysis showed that in group 1, the SCs were spread on all the surface of the molds, but not tridimensionally. In group 2, the SCs were observed only on the borders of the scaffolds, suggesting the migration of the host cells. The scaffolds developed in this study demonstrated fibrous and porous structure similar to the natural extracellular matrix of the cells and, therefore, promise to be a new biomaterial suitable for use in TE, due to its suitable characteristics for the culture of cells. This association of nanotechnology and stem cell culture is an innovative approach for producing a cutaneous substitute for burn patients.

Poster Board Number: F-2258

THE ADULT STEM CELL NICHE OF THE EARTHWORM, EUDRILUS EUGENIAE

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The earthworm, Eudrilus eugeniae, belongs to the family Eudrilidae. The first thirteen segments (anterior part) consist of mouth, simple brain, heart, testis, seminal vesicle, gut, ovary and skin. The segments from 13-18th are called as clitellum, which is important for the reproduction. The posterior part has prostate gland, gut, nephridia, skin and anus. The earthworm skin has three layers of cells: the outer most of them is called as epithelial cell layer (ECL) followed by circular muscle layer (CML); the inner most is named as longitudinal cell layer (LCL). The regeneration process starts with the formation of blastema. It has been reported by us that the strong fluorescent adult stem cells are in the boundary of ECL and CML, the major source of florescence is riboflavin and its derivatives; riboflavin augments regeneration. The blastema is produced by the cells of LCL during anterior regeneration, and then, the septum, which creates segments, is formed by differentiation of blastemal cells. Finally the organs are developed in the segments. In the present work, the followings were found: The amputation at 10th segment (anterior part) results two portions of a worm. The portion of segments containing clitellum produces blastema in 3 days; regains the mouth in 6 days; and ingestion starts from the 7th day. In contrast, the other portion separated from the clitellum fails to generate blastema. Similarly, clitellum detached posterior part, which has intact anus at one end, develops blastema on the 5th day, and forms anus instead of mouth at the amputated site on the 8th ±1 day, but blastemal cells could not be differentiated to form septum, and the blastema has been shredded off in 10±1 days. Then, the worm dies. In contrast, the portion of posterior part with clitellum forms blastema in 3 days, and regenerate the anus in 6th day, from which the excretion also starts. The data suggest that the paracrine support of clitellum is required for the proper regeneration of anterior and posterior segments. To confirm the data, the worms were amputated at clitellum. The resulted two portions of a worm have developed blastema on 6th ±1 day by the proliferation of cells of ECL instead of LCL, which has differentiated, and the septa have been formed, but the two worms die due to failure of further cell differentiation to form lost organs. Interestingly, two blastemas have been observed at an amputated site of clitellum, and the worms die in 20±3 days. The data confirm that intact clitellum is not only important for the faster & proper blastema development, but also for complete restoration of body parts, but the clitellum has capacity to regenerate its own tissues. Experiments were designed to find the mechanism, and they revealed that clitellum acts as reservoir for adult stem cells, which is the boundary of ECL & CML. Cells of CML differentiated into cells of LCL, and the adult stem cells migrate from clitellum to the site of

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blastema in either anterior or posterior segment, and the thickness of clitellum reduces significantly. Thus, the clitellum has key role in organs restoration and the E. eugeniae is valuable system to study the adult stem cell and regeneration biology. "First three authors contributed equally to the work"

Poster Board Number: F-2259

CELL FATE DETERMINATION UPON DNA DAMAGE INDUCTION AND TELOMERE DYSFUNCTION

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Accumulation of DNA damage plays a critical role in the onset of aging. Cells in our body are constantly challenged by DNA damage triggered by external factors such as radiations as well intrinsic stress generated by the cellular metabolism. When DNA damage cannot be efficiently repaired cells undergo an irreversible cell cycle arrest termed senescence or, alternatively die through apoptosis. DNA damage occurring in Stem cells is expected to have a major impact on tissue homeostasis. However, how do stem cells react to DNA damage activation remains poorly understood. To this end we have developed a mouse model in which DNA damage can be delivered exclusively to adult stem cells or differentiated cell types in an inducible manner. As a source of DNA damage we use acute telomere dysfunction triggered by depletion of telomere associated proteins. The resulting "uncapped" chromosome ends are recognized as sites of DNA damage and initiate a canonical DNA damage response. Using mouse models in which telomere deprotection can be induced in adult stem cells we are testing whether the alteration of these proteins contributes to DNA damage-induced aging by reducing the number of functional stem cells or, conversely, whether the persistence of damaged (stem) cells contributes to the reduced regeneration potential observed in aging organisms.

Poster Board Number: F-2260

HIGH THROUGHPUT STEM CELL DIFFERENTIATION

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Stem cells are a self-renewing source of differentiated cell types for use in cell therapy and drug discovery applications. However, high efficiency directed differentiation is technically challenging and currently a bottleneck in the field. Differentiation of stem cells to a mature cell type typically requires serial cell culture steps with sequential addition of growth and patterning factors. Testing a significant number of such differentiation protocols is therefore very labour intensive and time consuming and limits the development of optimised methods. We describe a high throughput combinatorial technology, Combicult™, that allows tens of thousands of differentiation protocols to be tested simultaneously. Combicult™ combines miniaturisation of cell culture on microcarriers, a pooling/splitting protocol and a unique tagging system to allow multiplexing of experiments. Bespoke bioinformatics software (Aridane™) utilises criteria such as hierarchical clustering and probability analysis to select optimal protocols for further validation. We present the results of several successful high throughput screens. Novel, serum-free protocols for the differentiation of embryonic and adult stem cells to somatic cells types relevant for cell therapy and drug development applications (e.g. osteoblasts, megakaryoctes, neurons and hepatocytes) have been discovered. In several protocols expensive growth factors and other variable components have been eliminated and replaced with small molecules. Furthermore, we demonstrate that Combicult can be used to discover protocols that are applicable across many cell lines. This is particularly relevant to the study of hES and iPS cells which exhibit significant variability between lines. Combicult™ can greatly accelerate the process of stem cell differentiation protocol discovery and has many applications. In particular, it efficiently enables: i) discovery of protocols for generation of cell types typically refractory to production from stem cells; ii) improved cell yields; iii) elimination of variable and expensive components; iv) investigation of stem cell biology and differentiation signalling pathways and v) cell line selection.

Poster Board Number: F-2261

SISTEMQC: A BROADLY-APPLICABLE MICRORNA-BASED MONITORING TOOL FOR STEM CELL QUALITY CONTROL AND DIFFERENTIATION MONITORING

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Stem cells derived from both embryonic and adult tissue or from reprogrammed somatic cells have significant promise for human regenerative medicine. However, despite similarities in developmental potential, several groups have found fundamental differences between stem cell lines that could impact on the potency and/or safety of the resultant cell populations but which were not predicted using current monitoring procedures based on flow cytometry and analysis of panels of mRNAs. There is a requirement for reliable tools to monitor cell populations during the processes of stem cell line development, directed differentiation and scale-up to safe, therapeutically-useful cell populations. Sistemic have developed a novel, sensitive, reliable, broadly-applicable monitoring tool that provides both a good indication of cell homogeneity and insights into underlying biological effects associated with any observed alterations in microRNA expression profiles . We demonstrate here that our approach also provides an assessment of the likely impact of the observed miRNAs changes on cell phenotype. We will present case studies to illustrate that SistemQCTM, provides a simple, robust and cost-effective tool to monitor the maintenance of pluripotentcy in stem cell lines across passages, the staging of directed differentiation from embryonic, iPS or direct reprogramming strategies and, post scale-up, an assessment of functional attributes and safety profile of the cells

Poster Board Number: F-2262

DIAGNOSTIC MICROBIOREACTOR ARRAYS FOR MULTIPLEXED MICROENVIRONMENTAL SCREENING OF PLURIPOTENT STEM CELL EXPANSION, MAINTENANCE AND DIFFERENTIATION

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The use of human pluripotent stem cells in regenerative medicine and drug screening is predicated on the ability to effectively direct both their undifferentiated expansion and differentiation into desired lineages. Exquisite control over stem cell fate is needed to efficiently produce sufficient, defined cell populations for such applications, yet this is substantially hindered by undefined culture components, signal crosstalk between multiple exogenous and endogenous factors, and spatiotemporal variations in microenvironmental composition inherent to conventional culture formats. We have developed scalable, valveless, continuous-flow microbioreac-

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tor arrays that both provide a full-factorial set of exogenous factor compositions (3 concentrations each of 3 soluble factors; 33 = 27 distinct conditions in total), and also allow controlled accumulation of paracrine factors. Using pluripotency maintenance (tracked by an EOS-GFP reporter line and *in situ* immunostaining) and mesendodermal differentiation (tracked by a MIXL1GFP/w reporter line) of human embryonic stem cells (hESCs) as examples, we demonstrate the unique ability of this platform to separate, visualise (at both population level and single cell resolution), identify and modulate paracrine effects that are not otherwise readily accessible. HES-3 hESCs were screened for maintenance of pluripotency markers against b-FGF and TGF-β1 in a chemically-defined medium background, with retinoic acid included as an internal pro-differentiation control. Factorial analysis revealed the main and interaction effects of the supplied factors on pluripotency marker expression, which was also strongly dependent on sequential position within a column of serial culture chambers, best explained by accumulation of paracrine factors that negatively modulate pluripotency. The microbioreactor array was then utilised to investigate differentiation of hESCs to a MIXL1+, primitive streak-like population. A MIXL1 gene reporter was activated in specific combinations of BMP-4, Activin A, and BIO (a canonical Wnt activator) treatment, and was dependent on the position within a column of serial culture chambers. Regardless of the factors supplied to cells, significant MIXL1 expression was only activated in downstream chambers within the device for certain conditions of supplied factors, suggesting accumulation of paracrine factors was required and direct action by BMP, Activin and/or canonical Wnt signals was not sufficient to activate robust expression. Modulation and identification of paracrine factors was then possible by screening putative paracrine factors or inhibitors of their signaling pathways Importantly, optimization of these culture conditions with the arrays was readily translatable to improving mesendodermal differentiation in conventional static culture protocols, exemplifying the immediate practicality of the microbioreactor array platform. This platform thus deciphers factor interplay and signalling hierarchies that control of stem cell fate, and is applicable as a universal microenvironmental screening platform for bioprocess optimisation, media formulation design, quality control for cellular therapeutics and cell-based drug toxicity and discovery.

Poster Board Number: F-2263

ABCD2 GENE IS DIRECT TARGET OF B-CATENIN AND TCF-4: IMPLICATIONS FOR THE THERAPY OF X-LINKED ADRENOLEUKODYSTROPHY

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X-linked adrenoleukodystrophy (X-ALD) is a peroxisomal disorder caused by mutations in the *ABCD1* gene encoding the peroxisomal ABC transporter adrenoleukodystrophy protein (ALDP). The induction of *ABCD2* gene, the closest homolog of *ABCD1*, has been mentioned as a target molecule for X-ALD therapy. However, transcription factors affecting expression of *ABCD2* gene are little known. Here, through in silico analysis, we found two putative TCF-4 binding elements between nucleotides -360 position and -260 position in the promoter of *ABCD2* gene. The transcriptional activity of *ABCD2* promoter was strongly increased by ectopic expression of β-catenin and TCF-4. In addition, mutations of TCF-4 binding elements by site-directed mutagenesis resulted in decreased promoter activity. Also, we demonstrated that β-catenin and the promoter of *ABCD2* gene were pulled down with β-catenin antibody through a chromatin immunoprecipitation (ChIP) assay. Moreover, qPCR analysis revealed that β-catenin and TCF-4 induce the mRNA levels of *ABCD2* gene in both the hematoma cell line and primary fibroblasts of X-ALD patient. Interestingly, we showed that the levels of very long-chain fatty acid (VLCFA) were decreased by ectopic expression of ABCD2-GFP as well as β-catenin and TCF-4. Taken together, our results demonstrate for the first time the direct regulation of the *ABCD2* gene by β-catenin and TCF-4, and this direct regulation may provide a new strategy to drug development for X-ALD. We are now further investigating this regulation and the levels of VLCFA in oligodendrocytes differentiated from childhood cerebral ALD (CCALD)-induced pluripotent stem cells (iPSCs). This research was supported by a grant (SC1110) from the Stem Cell Research Center of the 21th Century Frontier Research Program funded by the Ministry of Education, Science and Technology and a grant (2010-0020353) from National Research Foundation, MEST , Korea.

Poster Board Number: F-2264

GENE EXPRESSION COMMONS: OPEN PLATFORM FOR ABSOLUTE GENE EXPRESSION PROFILING

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Gene expression profiling using microarray has been limited to profiling of differentially expressed genes at comparison setting since probesets for different genes have different sensitivities. To compensate for that, conventional methods always provide relative difference after comparison of 2 or more samples. Thus even recent microarray integrated probesets for entire sets of known genes, absolute gene expression profiling of a particular sample has not been achieved. We tackled this limitation with the hypothesis that if we accumulate a very large number of microarray datasets as a common reference, meta-analysis could be applied to it to compute statistical attributions of each probeset such as dynamic range or threshold to distinguish high expression from low expression. Then mapping individual sample data against the common reference, absolute gene expression profiling could be achieved. Computer simulations revealed that if size of common reference exceed 2560 microarrays, the reproducibility and accuracy of meta-analysis become highly stable. Common references were established by meta-analysis of 11939 Affymetrix mouse 430 2.0 microarray datasets and 25229 Affymetrix human U133 Plus 2.0 microarray datasets. Meta-analysis revealed wide variation in the dynamic-range of probesets. Then the strategy is implemented in web-based platform named "Gene Expression Commons" (https://gexc.stanford.edu/). To demonstrate the potential of the Gene Expression Commons, we generated gene expression microarray datasets of 39 hematopoietic populations covering almost the entire quantal stages of mouse hematopoiesis, and have integrated into the system. A platform for absolute gene expression profiling has been established utilizing meta-analysis of large-scale microarray datasets in public domain. Since the Gene Expression Commons has intuitive Web-interface and is designed as an open platform, any scientist can explore gene expression of any gene, search by expression pattern of interest, submit their own microarray datasets, and design their own working models.

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Poster Board Number: F-2265

ENGINEERING CELLULAR HOMING AND MIGRATION TO ENHANCE IMMUNE AND STEM CELL-BASED THERAPEUTICS

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A critical obstacle to the effectiveness of cellular therapies is the inadequate long-term homing, retention, survival, and integration of transplanted regenerative cells into damaged tissues. Successful future therapeutic application will likely depend on improvements in these areas. By utilizing a synthetic biology approach to "program" useful functionalities into cells through genetic modification, we seek to enhance the homing of cells to sites of disease. Using immune cells as a test-bed for our cellular engineering efforts, we have discovered that an engineered G-Protein Coupled Receptor (GPCR) activated solely by a biologically inert small molecule metabolite clozapine N-oxide is sufficient to redirect the migration of neutrophils and T lymphocytes in cell-based assays as well as in a mouse model. Genetically modified T lymphocytes have been used in human studies for the last two decades, making them an ideal platform for the study of cell migration and homing. Further, we are extending our efforts to study a class of pathologies in which defects in cellular migration are strongly implicated: neurocristopathies, which are diseases involving defects in the development of tissues containing cells derived from the neural crest cell lineage. We have generated neural crest stem cells from patient-specific induced pluripotent stem cells and are working to both study defects in cell migration in cell-based assays as well as to investigate the effects of reconstituting migration in these cells through an engineered GPCR. We envision that our work will help us better understand the cellular basis of disease in neurocristopathies and aid in therapeutic efforts to treat such pathologies through gene therapy and cell-based treatments.

Poster Board Number: F-2266

PROTEOMIC DEFINITION OF NORMAL AND BREAST CANCER STEM CELLS SUB-POPULATIONS: DISCOVERY OF STEM CELL SPECIFIC PROTEIN BIOMARKERS

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Background/Aims Previous studies have demonstrated evidence for the existence of adult stem cells with in the normal breast tissues. It has also been postulated that alterations in these cells or their progenitors give rise to cancer stem cells. This study is aimed to identify and characterize normal and breast cancer stem/progenitor cells using proteomics approach. Methods We have preformed tissue digestion on normal breast samples and extracted the mammary epithelial cells. We further purified for the epithelial fraction by depleting lymphocytes, fibroblast and endothelial cells. Categorization was based on the epithelial cell adhesion molecule (EpCAM) expression. Fluorescence Assisted Cell Sorter (FACS) was used to further investigate the normal progenitor/ stem cells by labeling them with specific cell markers. From the EpCAMhigh fraction, the ALDH+, CD44high/CD24 low and ALDH+/CD44high/CD24 low (ALL) cells were classified as progenitor/stem cells. These groups were sorted and their self renewal ability was tested based on mammosphere formation. Cells were subjected to proteome analysis by 2-DE and LC/MS/MS. Results We observed high degree of homogeneity in global protein expression profiles from all three different cell populations (ALDH+, CD44high/CD24 low and ALDH+/CD44high/CD24 low. The ALL group is a mixture that represents all sub-population of stem/progenitor cells. Despite high similarities in their protein fingerprints, we have identified 6 protein spots that are expressed in both ALL and ALDH but not in CD44high. Similarly 6 protein spots were expressed in both ALL and CD 44high but absent in ALDH. Only 4 protein spots were uniquely expressed in ALL but not in both ALDH and CD44high indicating their potentials as stem/progenitor specific protein biomarkers. This information may help in the discovery of protein biomarkers for breast cancer stem cells and better our understanding of molecular alterations involved in breast cancer stem cells and how they interact with the surrounding microenvironment. Conclusion These findings indicate that these protein biomarkers may be essential for improved definition of breast cancer stem cells. The results highlighting the power of proteomics for discovery of biomarkers for breast cancer stem cells will be presented.

Poster Board Number: F-2267

EPIGENETIC AND GENOME STABILIZING MOLECULAR MECHANISMS OF LEVO-CARNITORS IN RELATION TO CRYOBIOLOGY AND STEM CELL RESEARCH

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Cryopreservation is the only reliable form of long-term storage of viable cells and tissues. Oxidative stress (OS), hypotonic shock response, mechanical injury due to ice crystal formation, altered extent of shape change, altered physic-chemical properties of cellular structures, Na^+/K^+-ATPase inhibition, caspase-3 activation, externalization of phosphatidylserine, alteration of mitochondrial membrane potential and DNA fragmentation and an ultimate apoptosis occurs during cryothermal and freeze-thaw processes. Systematic PubMed review delineates the epigenetic, re-pairing and genome stabilizing mechanisms of bi-phasic iron-chelating antioxidant L-Carnitine (LC) and its acyl congeners. The t½ LC is around 60hrs and the continuous supply of its precursors are required to maintain the physiological conc. of 85µmol/L. LC improves the level of spectrin/cardiolipin, increases NADPH+H and ATP. LC serves as a buffer for deleterious acyl-CoA, LC mitigates the toxic onslaught of xenobiotics including antibiotics and ammonia. LC reduces the expression of glial fibrillary acidic protein, iNOS, ubiquitin proteasome and caspase-3 markers of cell stress. LC increases the expression of p53, which plays a central role in differentiation, repair, OS-induced apoptosis, by acting upstream of mPT, followed by the release of apoptogenic factors. LC exists as a reservoir of LCs to replace oxidized FAs acids in membrane PLs, sequestrates Ca^{2+}, inhibits PLA_2, augments membrane repair, and to restore age-related membrane damages. LC increases the viability/quality and

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shelf-life of the cryo-preserved sperms, oocytes, extended stored apheresis platelets, RBCs by repairing cytoskeletal damage, inhibits neutrophil $O_2^{-[[Unsupported Character - ▪]]}$, CRP and xanthine oxidase, prevents LPO, and spares overall antioxidants defence. LC was able to neutralize the anti-proliferative effect of TNF-α and reduced the level of embryonic DNA-damage and apoptosis. LC anneals DNA-s-s-breaks, chromosomal aberrations, increases the poly(ADP-ribose) polymerase and HSP-70 in acute stress, LCs provides acetyl groups for histone, prevents thermal stress and preserve the histone-chromatin integrity. LC was reported to down-regulate and/or increase clearance of cytokines such as IL-1, IL-6, and TNF-α. IVM medium with LC increased oocyte maturation-cleavage by enhancing mitochondrial biogenesis. LC facilitates differentiation of the leukemic population yielding a number of atypical for the myeloid lineage and promoted neuronal and cardiac differentiation. LCs increases the formation of colony-forming unit-erythroid-colonies in cultures of fetal liver. LCs increased both haeme-oxygenase-1 (HO-1) mRNA and protein expression. HO-1 is induced by OS and shown to exert antioxidant and antiapoptotic activity. LC inhibits apoptosis by interaction with the Fas-ligand/Fas-receptor systems. LC increases mitochondrial anti-apoptotic factors BCL-2 and BCL-XL, these can inhibit Bax activation/cyt-C release, thus preventing apoptosis.

Poster Board Number: F-2268

STEM CELL-SUPPORTING COATINGS AND HYDROGELS FOR 2D AND 3D CELL CULTURE APPLICATIONS IN VITRO AND FOR STEM CELL-DELIVERY IN REGENERATIVE MEDICINE APPLICATIONS *IN VIVO*.

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Stem cells grow in specific niche milieus in the body. Lack of such a specialized environment hampers many tissue engineering applications with stem cells. The major problem is the quick disappearance of the cells from the defect due to migration or apoptosis in the often hostile environment. Our hydrogels could be used to create a suitable stem cell microenvironment and at the same time retain the cells at the desired location. While ECM protein-based hydrogels offer excellent biological activity, using synthetic hydrogels would be very attractive due to their known composition, absence of animal components, and reduced batch-to-batch variation. We have developed a synthetic hydrogel (ArtiCYT) that mimics the structure of natural ECM proteins, potentially offering the best of both worlds. ArtiCYT is an enabling technology applicable to a wide range of innovative cell culture products. Functionalisation with cell-adhesion promoting peptides is easily done and cells can be encapsulated in a one-step procedure. Therefore, we aim at developing custom-made (stem) cell-supporting coatings and hydrogels for 2D and 3D cell culture applications in vitro and for (stem) cell-delivery in regenerative medicine applications *in vivo*. Coatings (ArtiSURF) and hydrogels (ArtiGEL) were prepared from different types of ArtiCYT gelator molecules. All gelators share a common core structure consisting of a 1,3,5-cis cyclohexane tricarboxylic acid core. The rigid-core predisposes the arms towards self-assembly resulting in fiber formation and gelation. ArtiCYTs with different cell-adhesive properties were obtained by conjugating different peptidic arms to the three arms of the core structure. Adipose-derived stem cells were isolated from liposuction material and interaction with our coatings and hydrogels was assessed by several assays. Attachment was assessed by light microscopical observation, fluorescence microscopy after staining and by SEM. Proliferation was measured with WST-1. Functionality of the ADSCs on coatings and in hydrogels was demonstrated by differentiating the cells along different lineages, e.g. the adipogenic, osteogenic, neuronal and chondrogenic lineage. Differentiation was assessed by performing lineage specific stainings and biochemical assays. Different ArtiCYTs were applied as coatings or gels in well-plates. Cellular function and morphology of ADSCs could be maintained on the coatings and in the gels. Moreover, proliferation of ADSCs on the tested ArtiSURF coatings as assessed by WST-1 was equal or better compared to ADSCs on uncoated tissue culture plates (TCP). In addition, our coatings and gels supported differentiation of ADSCs along the adipogenic, osteogenic, chondrogenic and neuronal lineage. A striking example was the differentiation of ADSCs along the osteogenic lineage on ArtiSURF 25 and 34. The deposition of bone mineralisation crystals was highly increased on both coatings compared to uncoated TCP (ARS staining). Alkaline phosphatase activity in the cells was slightly higher (ArtiSURF 25) or equal (ArtiSURF 34) compared to bare TCP. Culturing of ADSCs on 2D-coatings or in 3D-hydrogels showed that our materials are excellent scaffolds for propagation and differentiation of ADSCs in vitro. Moreover, as previous studies have shown that our Articyt gels are injectable, they are very promising materials for the development of injectable stem cell-seeded scaffolds for the regeneration of damaged or lost tissues *in vivo*.

Poster Board Number: F-2269

MULTIPLEX DETECTION OF THE STEM CELL PLURIPOTENCY MARKERS OCT3/4, SOX2 AND NANOG- A MILLIPLEX® PLURIPOTENT STEM CELL PANEL

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The transcription factors OCT4, SOX2 and NANOG play a critical role in the maintenance of pluripotency in embryonic stem cells. Additionally, over expression of any one or a combination of these three factors has been reported in solid tumors from a variety of tissues. The presence of pseudogenes and differentially expressed protein isoforms, complicates the accurate detection of the physiologically relevant form of these proteins in stem cell research. Here, we report the development of a Luminex-based assay for the simultaneous, specific and sensitive detection of OCT4, SOX2 and NANOG protein levels in cellular lysates. The assay exhibits 100-fold greater sensitivity compared to western blot analysis. This sensitivity is of special importance for the detection of rare cancer stem cell populations. Application of the assay to a panel of human cancer cell lines from breast, lung and colon origin revealed SOX2 to be the most often expressed factor among these lines. Of the cell lines assayed, only one breast cancer cell line expressed detectable levels of all three transcription factors, suggesting a role for OCT4, SOX2 and NANOG in cancer stem cells of this particular cell line. In addition to the data presented on cancer cell lines, data obtained through the analysis of hESC and iPS will also be presented. These experiments demonstrate the utility of this pluripotent stem cell panel for the sensitive analysis of stem cells from a variety of lineages.

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Poster Board Number: F-2270

SITE-SPECIFIC GENE INSERTION OF TRANSGENE BY ADENO-ASSOCIATED VIRUS INTEGRATION MACHINERY

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The adeno-associated virus (AAV) preferentially integrates into a site on chromosome 19 (19q13.4), termed AAVS1. A trans factor, Rep protein replicates the AAV genome and also mediates the integration into the AAVS1 site. The inverted terminal repeat (ITR) at either end of the AAV genome is a cis element for AAVS1-specific integration. The p5 promoter that drives the transcription of the Rep protein also can direct AAVS1-targeted insertion. Both the ITR and the p5 promoter sequences include two consensus sequences, Rep binding site (RBS) and the terminal resolution site (trs) required for AAVS1-specific integration. The Rep protein binds the RBS and then nicks at the trs, which is the first event in the integration. The ITR or p5 sequence alone is sufficient for the site-specific integration. To examine the AAVS1-specific integration efficiency with the ITR or p5 sequence, we constructed plasmids harboring GFP and blasticidin resistance (bsr) gene where a cis element was placed upstream of the GFP/bsr expression cassette. Following co-transfection of HeLa cells with a GFP/bsr plasmid and a Rep plasmid, we extracted genomic DNA and examined by semi-quantitative PCR for AAVS1 specific integration of the GFP/bsr plasmid. We found that the p5 sequence directed the GFP/bsr cassette into AAVS1 approximately 1.5-fold more, compared to an ITR bearing plasmid. In addition, the p5 promoter sequence inserted in the reverse orientation appeared to efficiently join the downstream GFP/bsr cassette to the AAVS1 locus, and vice versa. These results indicate that the p5 promoter sequence can directionally insert a transgene into AAVS1. We are analyzing individual cell clones after co-transfection of a cis plasmid and a Rep plasmid by PCR to quantify the efficiency of the AAVS1-specific integration.

Poster Board Number: F-2271

VISUALIZING SPATIOTEMPORAL DYNAMICS OF MULTICELLULAR CELL CYCLE PROGRESSION

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The transition from G1 to S in the cell cycle is difficult to monitor despite the fact that the process involves the critical decision to initiate a new round of DNA replications. To overcome this problem, we have harnessed ubiquitination oscillators that control cell cycle transitions in order to develop genetically encoded fluorescent probe, "Fucci" (fluorescent, ubiquitination-based cell cycle indicator). To design the ubiquitin oscillators, we used "degron" (Cdt1(30/120) for SCFskp2 and geminin(1/110) for APCcdh1) and red or green fluorescent proteins. Fucci probe effectively label individual G1/G0 phases nuclei red and those in S/G2/M phases green, and those in G1/S transition yellow. To understand the fundamental principles that coordinate cell cycle progression with cell growth, differentiation, migration, and cell death, we are applying Fucci probes to a variety of biological samples. We successfully generated cultured cells and transgenic animals (mice, zebrafish, and *Drosophila*) constitutively expressing Fucci probes. The visualization of cell cycle behavior of individual cells within complex tissues presents an irresistible challenge to biologists studying multicellular structures. We have also improved Fucci probes to detect exact cell cycle time point with high resolution. For example, Fucci3 and Fucci4 can distinguish individual G1, S, G2, M phases with different colors.

Poster Board Number: F-2272

KISS AND FLEE: REVEALING SECRETS OF STROMAL CELLS

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Mesenchymal stromal cells (MSCs) provide a cell based delivery system for trophic factors to promote repair of damage due to trauma, disease, and aging as well as immunomodulatory activities to suppress damaging effects of inflammation, autoimmunity, and graft versus host disease (GVHD) that can cause rejection of transplanted organs and tissues. Stromal cells have been isolated from virtually all adult and postnatal tissues and organs, but fetal sources may be superior with respect to proliferation, efficient differentiation into connective tissue cell types and trophic activities. Fetal stromal cells are easily recovered from amniotic fluid that is obtained during routine amniocentesis. Cell cultures derived from amniotic fluid frequently show cells with long cytoplasmic extensions that are reminiscent of neuronal axons. These and other observations have raised the question of whether stromal cells generate neural lineages and possess differentiation potential that extends beyond that expected of stromal cells. If this is true, stromal cell therapies could generate unexpected and potentially unsafe outcomes following transplantation. Here, we compared the neurogenic potential of the stromal cells derived from amniotic fluid and neural progenitors derived from our validated line of human iChM5 iPS cells. We tested for neurogenic potential of several amniotic fluid derived stromal (AFS) cell lines, including widely used A1 cells and newly established AFS lines that satisfy widely accepted criteria for stromal cell identity. Immunofluorescence microscopy showed that iChM5 cells, but not AFS cells, were immunopositive for Oct4, Tra-1-60, Tra-1-81 and SSEA5, standard markers for human pluripotent stem cells. Aggregates of iChM5 cells and AFS cells were processed for differentiation by mitogen depletion in parallel suspension cultures. Only iChM5 cells formed rosettes of neural progenitors and differentiated into neurons and glia on the basis of immunostaining and transcript analysis with Taqman gene expression assays (ABI). In contrast to iChM5 cell aggregates, AFS cell aggregates attached to culture wares and dispersed away from the point of attachment without obvious change in cell morphology. These findings demonstrated neurogenic potential of iChM5 cells, but not AFS cells. Live cell imaging methods were used to determine the source of long cytoplasmic extensions in AFS cell cultures. Transgenic AFS cells expressing GFP or mCherry were cocultured in glass bottom growth chambers (#1.5) and stained with contrasting vital dyes (Molecular Probes) to better track cell movements and development of cytoplasmic extensions. Real-time Z-sweeps through 4 µm sections of multiple fields were acquired at 5-minute intervals with a DVCORE live cell imaging system (Applied Precision) fitted with a 20X oil immersion objective. Virtually all AFS cells in the lines tested in independent trials showed cell migration over distances of more than 100 µm at rates that varied with cell size. AFS cells developed elongated extensions following cell:cell adhesion and subsequent migration of the adherent cells in opposite directions. The bridge linking adherent AFS cells became increasing thin, but eventually broke and retracted toward the parental cell. Our results indicate that cytoplasmic extensions of AFS cells reflect cell distortion by pulling forces that contrasts with microtubule-mediated cell extension of axons in differentiating neural progenitors.

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INTEGRATION AND COLLABORATION ARE NECESSARY FOR THE ESTABLISHMENT OF REGENERATIVE MEDICINE-THE PROTEUS PROJECT

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To undertake regenerative medicine using pluripotent stem cells including human embryonic stem (ES) cells), research fields associated with cell culture, cryopreservation, cell induction, tissue engineering and cell transplantation must be integrated and academic-industrial collaboration promoted. Integration facilities the visibility of regenerative medicine and accelerates its development. Collaboration ensures the availability of a large volume of cells at low-cost and safe implementation. Our research team has developed various techniques and technologies for the above-mentioned integration and collaboration since 2000, which are described here. *For Cell culture,* Xylose-culture medium (together with BOURBON CORPORATION & Nissui Pharmaceutical Co.) and high porous ceramics (with Covalent Materials Corporation) were developed for maintaining an undifferentiated state and the micro-incubator Cell Home (with Bio Optical Inc.) as a new apparatus to save the incubator space; *For Cryopreservation,* the program freezer CRYOEMBRYO (with Air Water Inc.) was used to cool embryoid bodies and gene-map analysis was underwent for establishment of efficiency, simplicity and confirmation of cryoprotectant containing Rock inhibitor; *For Cell differentiation,* growth factors were employed for differentiation of hepatocytes, cardiomyocytes, pancreatic cells; low-molecule-weight molecules for pancreatic cells and hepatocytes; dynamic force culture method for cardiomyocytes; cell-to-cell interaction method from ES cells, which were induced into pigment cells to ES cells, which were interacted by the pigment cells and were differentiated into rhodopsin positive cells; embryoid formation method using the LIPIDURE®-COAT (with NOF CORPORATION); the FISHMAN cell sorting system using ICG (with On-chip Biotechnologies Co.) for purification of hepatocytes. *For Tissue engineering,* the scratch method using LIPIDURE-COAT was developed to control cell geometric adhesion. *For Cell transplantation,* submucous transplantation for hepatocytes or pancreatic cells and cardiomyocyte transplantation using latissimus dorsi muscle as a carrier were undertaken.

Poster Board Number: F-2274

HITS-CLIP AS A TOOL TO STUDY NEURAL STEM CELL RESEARCH

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Alternative splicing provides functional protein diversity and higher-ordered biological complexity. Alternative splicing patterns in each transcript are regulated by binding of RNA binding proteins (RNABPs) to regulatory sites in a tissue-specific manner and proper cellular context. Recent studies have begun to define splicing codes by machine learning tools and high-throughput biochemistry. One such a promising strategy is an unbiased genome wide analysis, HITS-CLIP (HIgh-Throughput Sequencing of *in vivo* UV-Cross-Linked Immuno-Precipitation) that has been used to map protein-RNA direct interaction sites *in vivo*. It has shown that a general feature of alternative splicing regulation mediated by RNABPs is that the position where they bind pre-mRNA is a determinant of whether alternative exons are included or excluded (e.g the best studied mammalian splicing factor, Nova RNA regulatory map). Here, we revealed that a Nova RNA binding map in which the position of Nova binding determines the outcome of exon inclusion or exclusion is stable throughout cortical development, however, their biological targets switch in proper cellular context. Additionally, we generate the RNA regulatory maps in neural stem cells using HITS-CLIP by focusing on the other RNABPs expressing in neural stem cells. We will discuss the post-transcriptional regulatory loops during the differentiation from neural stem cells.

Poster Board Number: F-2275

NSC-MEDIATED GOLD NANOPARTICLE PHOTOTHERMAL TREATMENT OF TRIPLE NEGATIVE BREAST CANCER

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Triple negative breast cancer (TNBC) is characterized by tumors that do not express estrogen, progesterone, or HER-2 receptors, making it a considerable clinical challenge despite as advances in surgical, radiation, and chemotherapy treatments. TNBC has an earlier age of onset and worse clinical outcome than other breast-cancer subtypes. Recently, gold nanoparticles (AuNPs) have attracted attention as an inert therapeutic technology that is insensitive to drug resistance mechanisms. AuNPs that accumulate in tumor foci can be used to locally convert near-IR light into thermal energy intense enough to destroy tumor cells. Currently, free AuNP deposition within tumor foci relies on leaky tumor vasculature and poor lymphatic drainage following intravenous injection. This passive AuNP distribution is limited in efficacy by its restricted tumor penetration due to dense matrices, outward fluid-pressure gradients and inefficient penetration of vasculature in hypoxic tumor regions. NSCs have demonstrated inherent tumor tropic properties in preclinical brain and metastatic tumor models, migrating selectively to invasive tumor foci, penetrating hypoxic tumor regions, and even traversing through the blood-brain barrier to access intracranial tumor foci following intravenous administration. We have recently demonstrated that HB1.F3 NSCs exhibit a strong tropism to TNBC in vitro and in metastatic TNBC mouse models. Here we hypothesize that NSCs loaded with AuNPs can be used to more effectively distribute the AuNPs throughout TNBC tumors increasing the efficacy of photothermal therapy relative to free AuNPs. The established, human clonal HB1.F3.CD NSCs used in this study are currently in the first in-human Phase I clinical trials to mediate enzyme/prodrug therapy in recurrent glioma patients. Using these clinically relevant NSCs, we have optimized an AuNP endocytosis protocol and confirmed efficient NSC uptake of the AuNPs via transmission electron microscopy and dark field imaging. NSCs loaded with AuNPs exhibit no significant changes relative to NSC controls in viability (≥97%), morphology, and directed migration to TBNC-conditioned media in vitro ($p>0.05$). Photothermal trials in vitro have confirmed that AuNP-loaded NSCs exposed to 810nm near-infrared light generate thermal energy sufficient to kill exposed cells as assessed by confocal microscopy of live/dead stained cells. *In vivo* studies are now underway to evaluate photothermal therapy using AuNP-loaded NSCs as a more effective, selectively targeted, and drug-resistant treatment option for TNBC.

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Poster Board Number: F-2276

USE OF LIPOPHILIC NEAR-INFRARED DYE IN WHOLE BODY OPTICALIMAGING OF ADIPOSED TISSUE DRIVED STEM CELLS HOMING FOR ISCHEMIC RENAL INJURY

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We performed an optical whole-body imagingtechnique for monitoring normal and adiposed tissue drived stem cells(ASCs) homing in vivo and *ex vivo*. To visualize in vivo cell homing, DiR labeled ASCs were injected intravenously into F44 rats. A recently developed nearinfrared IR lipophilic carbocyaninedye1,1-dioctadecyl-3,3,3,3 tetramethylindotricarbocyanine iodideDiRis used to safely and directly label the membranes ofASCs. DiR has absorptionand fluorescence maxima at 750 and 782 nm,respectively, which corresponds to low light absorptionand autofluorescence in living tissues. Cells were incubated with DiR 1X07 cells in 10-ml phosphate buffered saline PBS containing 3.5g/ml dye and 0.5% ethanol_ for 30 min at 37°C. Thereafter, cells were washed twice with PBS and the viability of labeled rat ASCs This allows us toobtain a significant signal with very low background level.A charge-coupled device (CCD)based imager is used fornoninvasive whole-body imaging of DiR-labeled cellhoming in intact(n=6)and left ischemic renal rats(ischemic time:45 min) with(NxLIR: n=6) and without (LIR :n=6)rtnephrectomy groups, respectively. Dir accumulated to Lt ischemic renal injury(NxLIR>LiR>intact).This powerful technique canpotentially visualize any cell type without use of specificantibodies conjugated with NIR fluorescent tag or loadingcells with transporter-delivered NIR fluorophores. Thus, invivo imaging based on NIR lipophilic carbocyanine dyesin combination with advanced optical techniques mayserve as a powerful alternative or complementation toother small animal imaging methods.This study demonstrated that ASCs can be tracked in vivo by using noninvasive DiR method and have pathotropic properties toward an ischemic lesion of the kidney in whole body.

Education and Outreach

Poster Board Number: F-2277

BATTLING STEM CELL TOURISM: EDUCATING PATIENTS ON STEM CELL TOURISM AND TRANSLATIONAL RESEARCH

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Stem cell tourism raises significant ethical concerns related to providing unproven and potentially dangerous treatments to patients. Several studies examining patient perceptions on stem cell tourism have shown distrust in their home nation's research and regulatory system seeing it as stagnant, full of red-tape, and unresponsive to the needs of patients and their families. Many commentators have argued that one way to quell stem cell tourism is to educate patients, primary healthcare workers, and the public so that they understand the associated risks and the potentially fraudulent nature of the industry. However, little data exists on what information regarding stem cell tourism is being conveyed by patient, professional and scientific organizations. Due to the online nature of the stem cell tourism market, we sought to examine internet websites of many patient disease groups and relevant scientific research societies for information on stem cell tourism. Website content was searched for a variety of topics, including information on the science of stem cells and regenerative medicine, stem cell ethics, and the phenomenon of stem cell tourism. In the area of stem cell tourism, we looked for information on a variety of issues, including: whether the phenomenon was introduced; that therapies offered by clinics were based on little or no scientific rationale and could result in significant risk to patients; the need to have regulatory and ethics review and approval; and an explanation of the translational stem cell research process. We found that although some websites contain information on stem cell biology and regenerative medicine, only 25% of the organizations surveyed had information on stem cell tourism and the clinical translation process. Moreover, the depth of the information and topics covered varied amongst organizations. For education to have an impact on stifling stem cell tourism it would help to provide consistent information. We further argue that educational material on stem cell tourism should focus on explaining the nature and challenges associated the translation of stem cell research. This should stress that the good scientific research takes time, and is needed in order for therapies to be safe, and have a greater chance at being effective. We recognize that simply providing more accurate scientific information will not necessarily result in the desired change in public attitudes and behavior (this is related to the rightly critiqued *science deficit* model). While recognizing the limits of education, we argue that greater educational efforts are needed on stem cell tourism and clinical translation because providing information has been shown to influence one's decision-making, and can help patients make better informed decisions; this is valuable in and of itself irrespective of whether providing information serves as an effective deterrent against stem cell tourism.

Poster Board Number: F-2278

EXCITING HIGH SCHOOL STUDENTS TO THE POSSIBILITIES OF STEM CELLS: IPS CELL TECHNOLOGY AND CONCEPTS IN THE HIGH SCHOOL CLASSROOM

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The stem cell as a functional, biological concept is relatively new. As such, it encounters some misconstruing in the media and public light, while at the same time, receiving little attention in grade-, middle-, and high-school curricula in the United States. This problem persists, even though thousands of research papers are published on adult, embryonic, and induced pluripotent stem cells each year. One program working to ameliorate this problem is the NSF GK-12 Program. Since its inception in 1999, the GK-12 Program has funded over 200 projects in more than 140 different universities throughout the United States and Puerto Rico. The IUPUI Urban Educators GK-12 Program, funded by the National Science Foundation, has provided a competitive fellowship to integrate my graduate research on iPS cells in the visual system into a high school classroom. As part of this program, 10 hours a week are spent in a local high school offering students and their teacher access to current and relevant scientific concepts. By engaging students in a research framework, the GK-12 fellow allows students a unique

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opportunity to ask questions and think scientifically about every day, real-world issues in health sciences and the environment. A major theme of my efforts was to provide exposure to stem cell research with the intention of interesting and preparing students for college success and future careers in science and health care. In the classroom, my research approach was initially introduced to 1) address pre-existing knowledge and misconceptions about stem cells and 2) stimulate discussion and questions related to the concept of induced pluripotency and it's applications for research, as well as to the possibility of research as a career interest. These lessons - including PowerPoint-driven discussions, fact sheets, and inquiry-based labs - were developed in consultation with their classroom teacher, and integrated applications regarding the nature of science and working in a laboratory to address Indiana's state education standards such as the Principles and Historic Perspectives of Chemistry, as well as Integrated Chemistry and Physics. In addition to complete lessons, a daily concerted effort was made to emphasize 1) college preparedness and expectations, 2) each student's success as a scientist, and 3) a biological context for chemistry concepts. Specific lessons, labs, and discussions throughout the semester use current examples within stem cell biology, biochemistry, cellular plasticity, and experimental design to address core and current scientific concepts, while emphasizing the feasibility of research as a career path, and the importance of college-planning for students in a high school chemistry and an integrated chemistry and physics class.

Poster Board Number: F-2279

PROMOTION AND EXPANSION OF PLURIPOTENT STEM CELL RESEARCH - STEM CELL SHARED RESOURCE FACILITIES

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The maintenance of human embryonic stem cells (hESCs) and their differentiation into multiple lineages offers unprecedented opportunities to investigate and understand the earliest stages of human development. Recent revolutionary developments in the ESC field have led to the discovery that forced expression of defined gene/protein factors in adult somatic cells results in their transformation into an ESC-like pluripotent stem cell state referred to as "induced pluripotent stem cells (iPSCs)". This technology allows the derivation of patient-specific stem cell lines without the onus of social, moral and ethical dilemma's associated with the creation of new hESC lines. Like ESCs, iPSCs can self-renew thus providing us with a potentially unlimited source of cells. They can be genetically modified to define gene function and differentiated into multiple lineages. Furthermore, derivation of iPSC lines from individuals prone to various diseases also provides us with the opportunity to understand the etiologies of many complex syndromes. A dedicated hESC/iPSC shared resource facility (SRF), henceforth referred to as the 'Core', can aid principal investigators with pilot and/or continuing stem cell projects. Our first main objective is to make available the latest developments in the field of ESC / iPSC biology to scientists. Currently we are exploring different iPSC technologies to aid in the development of transgene-free iPSC lines and induced pluripotent cancer (iPC) cell lines. However generating disease-specific iPSCs is just the first step in this process. The next step involves the differentiation of these cells into the diseased tissue-specific cells in order to recapitulate aspects of the disease. To facilitate the latter, the Core, is involved in generating various reporter systems. These reporters will allow us to separate desired populations and establish conditions necessary to improve the efficiency of generating specific lineages. In addition, these reporter cell lines will also allow siRNA, micro RNA, small molecule/drug screens in novel cell populations. The second main objective of the Core is to quality control stem cell lines, reagents. These quality control services will include karyotyping, mycoplasma testing of hESC, iPSC and iPC cell lines as well as the supply of these cell lines and differentiated lineages to the interested scientific community (MTA permitting). Scientists are also provided with tested stem cell reagents at vastly discounted pricing made possible due to the establishment of two stem cell supply centers, bulk purchasing and NYSTEM funding. Last but not the least, the third main objective of the Core is to continue to conduct classes to teach iPSC generation and hESC/iPSC/iPC cell differentiation into the lineage of choice. The goal of this education is to increase in the number of labs that are able to carry out stem cell research independently. Taken together, these services will have a three-fold benefit. Firstly, it will alleviate the quality control burden of individual scientists and allow them to concentrate on important scientific questions. Secondly, it will allow collaborative projects involving hESC / iPSC /iPC cell lines to be initiated with multiple laboratories by removing the prohibitive cost and providing the expertise required to establish and sustain this technology and lastly it will provide the Core with a source of revenue to meet its expenditures.

Ethics and Public Policy

Poster Board Number: F-2281

IRRATIONAL EXUBERANCE IN THE MARKETING OF PRIVATE STEM CELL BANKING SERVICES

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The number of commercial services that offer the private storage of umbilical cord blood and other tissues enriched for hematopoietic and other stem cells has grown continuously since the first such bank was established nearly thirty years ago. Today, approximately 150 private cord blood banks are in operation worldwide, and the clinical use of cord blood in the treatment of diseases of the blood and immune systems has expanded rapidly over the past decade. Nonetheless, the industry has frequently been criticized, particularly with regard to the marketing strategies used to promote cell and tissue banking services on a direct-to-consumer basis. Many companies advertise their services as a form of "insurance" for the newborn and their families, with support from epidemiological studies that seek to calculate likelihood of the clinical need for a cord blood transplant over the life of an individual based on current trends. However, these marketing efforts do not equally highlight the degree of uncertainty surrounding the sustainability of the private cord blood banking business model over a similar timeframe. It is demonstrably less certain that private banks will remain viable, specifically with regard to regulatory climate and financial sustainability, for the multiple decades of life on which these usage projections are based, or that scientific progress will favor the clinical use of cord blood-derived stem cells over alternative, possibly unpredicted, future developments. In this study, we have analyzed marketing materials from leading private cord blood banks in the United States and other countries to evaluate the degree to which such uncertainties are explicitly acknowledged. These findings may be of value in informing the decisions of prospective users of private stem cell banking services, and of policymakers.

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ESTABLISHING A SYSTEM FOR DERIVATION AND DISTRIBUTION OF THE HIGH QUALITY HUMAN EMBRYONIC STEM CELL LINES

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Background: Eight human embryonic stem cell (hESC) lines are available in Japan. In order to conduct clinical trials which evaluate the safety and efficacy of cell therapy in patients with hESCs, high quality cell lines applicable to patients must be derived. The present study aimed to establish a system which included procedures for the procurement of spare preimplantation embryos and hESC distribution to ensure proper practice by scientists and staff members. Methods: We assembled a task force composed of investigators in research institutions, medical staff in hospitals who provided human gametes and embryos, and individuals including women who had experience of receiving infertility treatment. This task force met to discuss ethical and social issues surrounding hESC research, according to governmental regulations and international guidelines. We formed committees and developed documents and tools according to generally accepted systems in clinical trials for new drug developments. Results and conclusions: We developed procedures for spare blastocyst procurement, consent forms, a DVD explaining hESC research, and standard operating procedures. An oversight committee was also established to review how the overall process functioned in relation to the institutional review boards. We also formulated our own guidelines that articulate the mission and core values that would be shared by scientists engaging in hESC research, as the existing guidelines are incomplete in that they do not stipulate the specialized practices that vary by locality and region. We determined that informed consent should be obtained from blastocyst donors and hESCs are distributed with the caveat that researchers accept these principles and academic and non-academic institutions adhere to the guidelines. To establish a system for proper practice within the research community, bioethicists must facilitate collaboration by encouraging researchers to take a proactive role in this and provide an ethical framework to ensure that hESC research is conducted responsibly.

Poster Board Number: F-2283

DEFINING "RESEARCH" IN THE US AND EU: CONTRAST OF "SHERLEY V SEBELIUS" AND "BRUSTLE V GREENPEACE" RULINGS

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In 2011, courts in both the United States and European Union released decisions related to human embryonic stem cell (hESC) research. In both cases, the courts reviewed the definition of research: does it entail every step in the research process including the derivation of hESC cells, or focus solely on the set of experiments presented, which used existing cell lines? In the United States, the US District Court of Appeals, in the case "Sherley v Sebelius," defined research as a specific and finite project or group of experiments, independent of any prior research. This allowed for federal funding of research utilizing hESCs but not their derivation. In contrast, the EU case, "Brüstle v Greenpeace," focused on the issue of patenting technology linked to hESCs and defined research as an entire body of work comprised of both previous and proposed studies. The European Union forbids the patenting of technologies that commercialize embryos as this violates the moral code. Therefore, the Court of Justice of the European Union determined inventions related to hESCs were unpatentable since they resulted from research that utilized human embryos. Here we will describe the progression of both court cases and their impacts on scientific research and development. We will also compare how they fit within the current policy frameworks and ethical standards in the United States and European Union. In addition, we will explain how the varying definitions of "research" have developed, affected federal stem cell policies, and impacted stem cell research and commercialization. Overall, we determine that the courtroom is an inefficient and inappropriate place to set federal policies regarding scientific research - although it is quite common, especially in the United States. These entities lack the scientific knowledge and expertise to rule on these issues. Ideally, these policy decisions should be made within scientific governmental agencies with advice from experts in the field as well as input from ethicists familiar with the science. In this way, ethical policies can be instated that keep in mind the best interests of researchers and the general public.

Poster Board Number: F-2284

FUNDING EMBRYONIC STEM CELL RESEARCH IN EUROPE IN LIGHT OF BRUSTLE V GREENPEACE

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Patents are seen as essential for the development of new technologies as the granting of a patent gives patent holders a 20 year monopoly to exploit their invention which provides an economic incentive to invent new products. Due the high costs associated with the research and development of new technologies and products, without this economic incentive it is unlikely that companies would be unwilling to invest funds into the development of new technologies. As the first embryonic stem cell line was derived using funds from a private company, embryonic stem cell research is one such technology which has benefited from private funding. However in October 2011, the European Court of Justice ruled that if an embryo is destroyed at any stage of the making of an invention, that invention may not be patented under Article 6(2)(c) of Directive 98/44/EC on the Legal Protection of Biotechnology Inventions. In the immediate aftermath, the decision was decried as being bad for science as it will lead to the removal of funding for embryonic stem cell research in Europe and result in the ceasing of embryonic stem cell research in Europe. However a closer look at the patent industry suggests that all may not be lost for European stem cell research. This paper will analysis the impact that the Brustle decision is likely to have on embryonic stem cell research in Europe. First it will consider the decision and outline the main points of the decision. Second it will discuss the patent industry and its link with the funding of science. Third this paper will highlight the negative impact that the patent industry can have on scientific progress and the limits that the patent industry may potentially put on embryonic stem cell research in the United States. Fourth this paper will argue that due to the high level of public funding of embryonic stem cell research in Europe, the Brustle decision may not be as devastating as first thought. Finally this paper will conclude that the regulation of stem cell research at a European level should not be left to the patent industry but should be part of a coherent system which considers the legal, ethical and scientific issues relevant to embryonic stem cell research.

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Poster Board Number: F-2285

HIGHLIGHTS AND THEMES FROM THE QATAR INTERNATIONAL CONFERENCE ON STEM CELL SCIENCE AND POLICY

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Since 2007, the Qatar Foundation for Education, Science and Community Development has worked with the James A. Baker III Institute for Public Policy at Rice University to develop the Baker Institute International Stem Cell Policy Program. This program analyzes stem cell research, policy and ethics in an international context. To this end the Qatar Foundation, in collaboration with the Baker Institute, has hosted two conferences in Doha, Qatar to convene world leaders in stem cell research and policy. At the first conference in 2009, participants reviewed current stem cell research and regulation. The aim was to guide Qatar as it developed policies compatible with its cultural, religious, and ethical standards. The second conference, in February 2012, expanded previous discussions on policy, ethics, and research during the three day event "Qatar International Conference on Stem Cell Science and Policy." The goal of the 2012 conference was to inform and engage scientists in an array of stem cell issues as well as bridge the gap between science and policy in an international context. Science sessions covered pluripotent, hematopoietic, and cord blood stem cells; stem cell transplantation; and the uses of stem cells in the treatment of cardiovascular disease and neurological disorders. In addition, ethics and policy discussions were organized to address embryonic research and egg donations, stem cell banking, international regulation of stem cell research, and clinical trial oversight. There were also panels that specifically highlighted stem cell research and policies in the Middle East region. While a major aim of the conference was to bring attention to Qatari's stem cell research program, it was also an opportunity for regional scientists and ethicists to discuss their religious and cultural views of this research. Furthermore, we believe the event helped encourage international collaborations, especially between scientists in Qatar and visiting researchers. This article will highlight different aspects of the conference proceedings including novel stem cell research, current ethical discussions, and pressing policy issues that are region-specific. We will also relate the major themes of the conference and offer an outlook on the future of stem cell research and its obstacles both in the Middle East and globally. Finally, we will offer recommendations for sustaining international meetings that encompass broad issues in this important area of cutting edge science.

Poster Board Number: F-2286

NATIONAL REGULATIONS AND HARMONIZED GOVERNANCE OF STEM CELL BANKING

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The international nature of stem cell research demands that facilities exist to house stem cell lines that can be accessed by researchers around the globe. In light of this international demand, stem cell banks must establish consistent operating procedures and regulations that ensure safely and ethically sourced and maintained stem cell lines. There is general consensus that the development and use of stem cell banks on an international level necessitates harmonization of both internal and external regulation. Currently a "patchwork" of policies and regulations exist creating confusion and inconsistencies that could impede the advancement of stem cell research. Institutional efforts like the International Stem Cell Forum's (ISCF) International Stem Cell Banking Initiative (ISCBI), UMASS International Stem Cell Registry (ISCR) and the European hESC Registry have gained significant support within the stem cell research community. These institutions have worked to establish consensus on principles for standard operating procedures and guidelines for the procurement, storage, maintenance and transport of stem cell lines. The aims of these initiatives are to establish a globalized network of access to reliable stem cell lines, however it remains unclear what role legal regulation does or should play in conjunction with these efforts. This study will identify, review and assess the current laws and regulations that affect stem cell banking in selected jurisdictions e.g. Canada, the US, the European Union, Australia, and Japan. The scope will include legislation and policies governing banking and the use of human tissue, as well as the approval and oversight of stem cell-based products. It will examine how these laws and regulations relate to the organizational efforts of current institutions to harmonize banking standards. The ways in which harmonized standards can co-exist with or be given binding effect by regulations will be highlighted. The objective is to provide a clearer picture of the role that regulation can play in the governance of stem cell banking.

Poster Board Number: F-2287

STIMULATING ADULT STEM CELL THERAPY IN THE NETHERLANDS 3RD YEAR UPDATE

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Stem cell therapies are frequently promoted on the internet as the answer to serious clinical conditions. The ISSCR expresses its concern that these generally costly therapies have neither been proven to be safe nor effective. Still they are sold to patients suffering from these conditions. So there is a clear need for sound translational research on safety and effectivity of potential stem cell therapies. The Netherlands Organisation for Health Research and Development (ZonMw) supports exactly this type of research in the Translational Adult Stem Cell Research Programme (TAS). Projects aim at performing a clinical phase I/II trial within six years. This is to ensure the focus of the project to be on translation and milestones are set accordingly. So far 14.3 million euros have been allocated to 11 Translational Adult Stem Cell Research projects. In addition to funding, the programme with a total budget of 23.5 million euros supports individual projects to facilitate translation of preclinical results to the clinic. On the programme level workshops are organised on general subjects like regulatory hurdles in translation in the national context. On the project level specific phases (preclinical, farmaceutical, clinical) make up the committed project format to optimally support the translational process. Furthermore, user committees are installed for each project to provide a wide range of translational expertise. These users committees have yearly meetings to discuss progress with external experts and stakeholders and to support the project leader with expertise with respect to the further implementation of the project results. Honoured TAS projects are diverse in terms of stem cell type applied and disease addressed. They are promising to contribute to the development of new treatments for various serious diseases. The TAS programme provides a framework for these projects to guide preclinical stem cell research into appropriate clinical research. This is a prerequisite for the responsible development of adult stem cell therapies.

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Poster Board Number: F-2288

THE DISCLOSURE AND MANAGEMENT OF RESEARCH FINDINGS IN STEM CELL RESEARCH AND BANKING

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A complex and polarized debate has evolved over the rationale for disclosing research findings (i.e. general, individual or incidental) to participants. Seemingly, consensus has emerged over an ethical "duty" in certain contexts to disclose qualified research findings to participants in genetics and genomics studies. The essential conditions for such disclosure are clinical and analytical validity, clinical utility and actionability. This debate has been prompted by an increased interest of both research participants and the patient advocacy community in obtaining information about research outcomes and on the use of their biological samples. Furthermore, the use of new technologies (e.g. whole genome and exome sequencing, SNPs arrays) reveals both genetic data and significant amounts of incidental findings with possible clinical significance. These technologies together with the proliferation of biorepositories have further provided a compelling rationale for governments and scientific institutions to adopt prospective policies. The context in which research findings are generated has shaped the policy debates and outcomes. Indeed, it is the particular research context that determines whether an ethical - or even a legal - duty could or should be established. Given the scarcity of policies in the context of stem cell research and banking, a discussion on the scientific, ethical and legal implications of disclosing and managing research results for research participants is needed. Any policy approach must take into account the vast range of pluripotent stem cell research related studies and the sizeable diversity of banking initiatives wherein a wide range of pluripotent stem cell lines are continuously immortalized, transformed and distributed. The latter should be placed in a context where cell line misidentification continues to be a pervasive problem, altering the extent and authenticity of certain research findings. We will present for discussion and comment the recent International Stem Forum (ISCF) Ethics Working Party's Policy Statement on the Disclosure and Management of Research Findings in Stem Cell Research and Banking.

Society Issues

Poster Board Number: F-2289

A NETWORK FOR STEM CELL RESEARCH AND REGENERATIVE MEDICINE IN GERMANY: THE GERMAN STEMNET INITIATIVE

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Regenerative therapies rely on the availability of suitable pluripotent and somatic stem cells. Moreover, stem cells are not only critical during development, but are also involved in the etiology of several diseases and can be used for novel innovative approaches for drug testing. Full exploration of the enormous potential of stem cells for novel clinical therapies requires the interaction and communication between researchers, clinicians, stakeholders and the general public. International and national networks of scientists allow advanced interaction among groups of scientists and funding agencies, policy-makers, media, patient groups, teachers and the general public. The ISSCR coordinates these initiatives at an international level. However, it is critical that these efforts are supported by well organized national networks which help to coordinate academic and non-academic stem cell research, development of medical standard operating procedures, protection of patient rights, development of future technologies as well as inform and educate the public about stem cell related technologies. Here, we propose the new German stem cell network called "German StemNet" that will be founded by eleven national institutes for stem cell research and regenerative medicine as well as the ministry of research and education (BMBF). The major aims of German StemNet are: - Establishment of an organizational structure which coordinates Stem Cell related issues in Germany - Establishment of a central office in Berlin - Planning and conducting an international "German StemNet Annual Conference" starting in 2013 on stem cell research and regenerative medicine - Launch of a web-based platform to communicate the newest results and related information about stem cells and regenerative medicine in German and English. Moreover, this platform will be used for discussion and networking between national and international scientists, institutions, policy-makers, media and the general public - Establishment of subject groups on different topics in stem cell research, i.e. reprogramming and pluripotency, somatic stem cells, stem cells in diseases, stem cells in tissue engineering, regenerative therapies as well as drug testing. - Publish newsletters and print products on stem cell research and regenerative medicine in Germany - Establishment of an expert scientist database for scientific communication with media, schools, politicians, patient initiatives and other interest groups - Communication with science outreach initiatives, e.g. patient groups, technology museums, local science days, and organizers of science events etc. Comments and suggestions in particular from other national stem cell networks are highly welcome since the German StemNet has the aim to closely interact and exchange information with other national networks in addition to the ISSCR.

Poster Board Number: F-2290

SHOULD CELL THERAPY PRODUCTS BE DRUGS OR DEVICES IN JAPANESE REGULATIONS?

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Japanese regulation system for medical products has two categories, drugs and medical devices, under the Pharmaceuticals Affairs Act (PAA). Thus, cell therapy products regulated by the PAA in Japan will be classified as drugs or medical devices according to their characteristics. If the product acts mainly pharmacologically, it will classified as a drug, if physically act, a medical device. In the United States, the FDA has third category, biologics, in their regulatory system and they are regulated as biologics. Their regulation for biologics is similar to that for drugs. On the other hand, the European Medicines Agency announced on 2007 that the advanced therapy medicinal products including cell therapy products will be regulated by the Agency, that means they will be regulated as drugs rather than devices. In my presentation, I will discuss what is the better way to regulate cell therapy products in Japan.

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Regeneration Mechanisms

Poster Board Number: F-2291

DECLINE OF TRANSPLANTED NEURAL STEM CELL IMMUNOMODULATORY FUNCTIONS WITH TIME

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Fetal neural stem/precursor cells (NSC) possess powerful immunomodulatory properties by which they block multiple inflammatory signals, reduce production of pro-inflammatory cytokines, and inhibit immune cell activation, proliferation, and function. These characteristics enable them to attenuate neuroinflammation and protect the brain from immune-mediated injury. The immunomodulatory effects of NSCs are therefore regarded as key therapeutic targets of stem cell transplantation. A major issue in the development of stem cell therapy for chronic neurological disorders, such as multiple sclerosis, is whether cells maintain their immune-regulatory properties for a prolonged period of time. Long term stable preservation of these properties may allow allogeneic stem cell grafts to evade rejection from the host brain and to continue down-regulating neuroinflammation. Therefore we examined here whether intracerebrally-transplanted NSCs are able to inhibit early versus delayed induction of autoimmune brain inflammation and to inhibit an allogeneic rejection reaction against the graft. Following transplantation, 80% of transplanted NSCs remained in an undifferentiated state. However, allogeneic fetal NSC grafts elicited a strong immune reaction and were rejected from the host brain. Notably, there was strong upregulation of MHC-I expression in transplanted cells, rendering them visible to the immune system. Massive infiltration of the graft by activated microglia and T cells was observed within two weeks after transplantation and complete absorption by two months. Then, we examined in two experimental paradigms the ability of syngeneic intraventricular fetal NSC grafts to attenuate brain inflammation during experimental autoimmune encephalomyelitis. NSCs inhibited efficiently acute brain inflammation. However, transplanted NSCs lost their therapeutic effects with time and failed to inhibit relapses that were induced in a delayed manner (4-6 weeks after transplantation). In correlation, long term cultured NSCs lost their capacity to inhibit immune cell proliferation *in vitro*. We conclude that long-term functional changes in transplanted NSCs lead to loss of their therapeutic immune-regulatory properties, and render allogeneic grafts vulnerable to immunologic rejection. Thus, the immunomodulatory effects of NSC transplantation are limited in time, and one may be required for repeated injections in clinical use.

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NEURAL STEM CELL GRAFT FATE IS DEPENDENT ON A NEUROGENIC BRAIN ENVIRONMENT DUE TO DECLINE IN THEIR NEUROTROPHIC PROPERTIES

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Neural stem / precursor cell (NSC) possess powerful immune-regulatory and neurotrophic properties by which they can facilitate the self repair capacity of the adult central nervous system. The therapeutic value of NSC therapy in neurodegenerative diseases is critically dependent on their long term functional properties. However, while the multileveled beneficial effects of transplanted NSCs have been well characterized in the acutely-injured brain, it is not clear whether transplanted NSCs survive in the host brain and maintain trophic properties for extended periods of time. An important aspect of NSC neurotrophic properties is their ability to support their own survival independent of any exogenous growth factor. Therefore, we examined transplanted NSC survival in different regions of the healthy and injured brain. Survival of E13 fetal mouse brain GFP+ NSC spheres was quantified at 2 months after transplantation to naïve mice. Substantial survival of NSC grafts was observed in the ventricles, 70±16% survival in the hippocampus, but only 17±3.7% survival of striatal grafts (P=0.008, as compared to hippocampus). Brain irradiation abrogated neurogenesis in the dentate gyrus subgranular zone. NSC spheres that were transplanted into the hippocampus of irradiated mice exhibited markedly reduced survival (7±9%, P=0.003). In 6-hydroxydopamine lesioned striata there was strong induction of CD31, β1-integrin and GFAP expression, as well as multiple nestin+ and BrdU incorporating cells. NSC transplantation into 6-hydroxydopamine lesioned striatum resulted in marked improvement in graft survival (52.4±6%, P=0.0005 as compared to naïve striatum). Characterization of graft cells at 2 months post transplantation in the non-irradiated hippocampus and lesioned striatum showed less than 5% nestin+ cells, no β1-integrin+ cells and only 16% acquired mature markers. In view of the central role of β1-integrin in maintaining the adult subventricular zone stem cell niche, we examined its role in supporting transplanted NSC survival. Anti-β1-integrin blocking antibodies were continuously delivered intraventricularly for 28 days after NSC transplantation into the 6-hydroxidopamine lesioned striatum. Neutralization of host brain β1-integrin caused a 73% reduction in graft survival (P=0.016). In correlation with the *in vivo* findings, long term cultured NSC spheres showed a dramatic decline in proliferative activity, a 3-fold increase in TUNEL+ apoptotic cells and significantly reduced neurotrophic factor secretion. Long term cultured NSCs had diminished effects on PC12 neurite extension and on oligodendrocyte progenitor cell proliferation and differentiation as compared to fetal NSCs. Thus, transplanted NSCs lose their trophic properties with time and do not support their own survival, becoming dependent on environmental cues of brain neurogenic niches. Damaging these niches compromised graft survival, whereas induction of a neurogenic environment resulted in significant improvement of graft survival. The site dependent graft survival and time-limited therapeutic effect have important implications for designing cell therapy in neurodegenerative diseases.

Poster Board Number: F-2293

BASIC STUDIES FOR THE DEVELOPMENT OF A LESS INVASIVE LIVER REGENERATION THERAPY USING THERMOREACTIVE ORGANIC/INORGANIC NANOCOMPOSITE GELS-CULTURED BONE MARROW DERIVED CELLS

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Background and Objectives: We have previously demonstrated the efficacy and safety of autologous bone marrow cell infusion (ABMi) therapy for decompensated liver cirrhosis patients in a multicenter clinical trial. However, this therapy involves bone marrow (BM) aspiration under general anesthesia, and is not indicated for patients for whom general anesthesia is difficult. We therefore aimed to develop a new liver regeneration therapy in which cells showing curative effects for liver cirrhosis are isolated from a small amount of autologous BM aspirated under local anesthesia and cultured on

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the surface of thermoreactive organic/inorganic nanocomposite gels (NC-Nano) before infusion back into the same subject. Methods: We screened 40 kinds of NC-Nano-coated dishes based on the number of cultured murine or human BM-derived cells under 10% fetal bovine serum or completely serum-free conditions at days 7 and 14. Cultured cellular characteristics were analyzed by a flow cytometer and DNA-chip analysis. To assess effects on liver fibrosis, cultured murine BM cells on thermoreactive nano-materials were infused into mice with carbon tetrachloride-induced cirrhosis via a tail vein. Liver fibrosis was assessed by Sirius red staining. Results: We developed two novel thermoreactive NC-Nanos (MS114N1 and MC114N7) showing significantly higher numbers of murine and human cultured BM cells on these dishes even in serum-free medium compared to controls. Cultured cells were easily detached simply by changing the temperature to room temperature for 15 minutes without trypsin solution. Flow cytometric analysis revealed murine CD45 (95.1%)/CD11b (98.6%)-positive and CD90-negative cells, and human CD73 (99.2%)/CD105 (94.4%)-positive and CD45/CD11b-negative cells grew on these nano-materials. Ingenuity Pathway Analysis also showed higher expressions of pluripotency genes (such as oct4, pax6, otx1, and lhx5) in human cultured BM cells on the MC114N7 surface at days 30, consistent with showing the differentiation into adipocytes, osteocytes and chondrocytes under adequate differentiation conditions, compared with those on non-coated normal dishes. Moreover, transinfused murine green fluorescent protein (GFP)-positive BM cells on serum-free MS114N1 dishes showed a significantly decreased Sirius red-stained area (Control, 6.0%; MS114N01, 3.7%; $p<0.01$), consistent with the repopulation of GFP-positive BM cells expressing MMP-9 into the damaged liver. Conclusions: These results demonstrate the possibility of a new liver regeneration therapy for decompensated liver cirrhosis patients using infusion(s) of effective cells derived from a small amount of BM fluid and cultured using a thermoreactive NC-Nano-coated dish.

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BONE MARROW CELL INFUSIONS SUPPRESS HEPATOCARCINOGENESIS IN N-NITROSODIETHYLAMINE-AND CARBON TETRACHLORIDE-INDUCED HEPATOCARCINOGENIC MICE WITH LIVER CIRRHOSIS

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Background and Objectives: We have previously demonstrated the efficacy and safety of autologous bone marrow cell infusion (ABMi) therapy for decompensated liver cirrhosis patients without hepatocellular carcinoma in a multicenter clinical trial. However, as liver cirrhosis is highly oncogenic, evaluation of the effects of ABMi on the mechanisms of hepatocarcinogenesis is of great importance. We therefore performed frequent bone marrow cell infusion (BMi) in hepatocarcinogenic mice with liver cirrhosis, and analyzed effects on hepatocarcinogenesis. Methods: The DEN/GFP-CCl4 model was developed by intraperitoneally administering N-nitrosodiethylamine (DEN) once to 2-week-old male C57BL/6 mice, followed by repeated twice-weekly intraperitoneal administration of carbon tetrachloride (CCl4) from 1 month later, with these mice serving as the control group (Ctrl; n=17). In the administration group (BMi; n=23), GFP-positive bone marrow cells (1 ×106 cells) from syngeneic mice were infused via a tail vein in DEN/GFP-CCl4 model mouse biweekly from 2 months after DEN treatment. Kinetics of hepatocarcinogenesis were histologically evaluated at 4.5 months after DEN treatment (a total of 5 bone marrow cell infusions) based on the incidence, number, and size of foci and tumors (adenoma + hepatocellular carcinoma). Liver fibrosis, hepatic 8-hydroxy-2-deoxyguanosine (8-OHdG) levels, hepatic SOD activity, and expression of erythroid 2 p45-related factor 2 (Nrf2) were also assessed. Results: At 4.5 months after DEN treatment, foci in the BMi group showed significantly lower incidence (Ctrl, 70.1%; BMi, 17.4%; $p<0.001$) and smaller number (Ctrl, 2.01/cm2; BMi, 0.61/cm2; $p<0.01$), while size was almost equal (Ctrl, 0.51 mm2; BMi, 0.28 mm2; $p=0.35$). In addition, tumors in BMi livers also showed lower incidence (Ctrl, 64.7%; BMi, 30.4%; $p<0.05$) and smaller number (Ctrl, 2.07/cm2; BMi, 0.62/cm2; $p<0.01$), without significant difference in size (Ctrl, 4.70mm2 ; BMi, 3.50mm2; $p=0.43$). No GFP-positive tumor was found in BMi livers. Moreover, BMi livers showed significantly reduced Sirius red-stained area ($p<0.05$), consistent with significant lower 8-OHdG levels ($p<0.01$), higher SOD activity ($p<0.05$), and increased nuclear translocation of nuclear factor-Nrf2. In addition, there were higher numbers of SOD3-positive cells in BMi surrounding livers, and many SOD3-positive-cells were also positive for GFP protein on immunohistochemical analysis. Conclusions: These results demonstrate that frequent BMi might contribute to suppressed tumor initiation during stages of hepatocarcinogenesis, consistent with improvements in liver fibrosis and stabilization of redox homeostasis directly, suggesting a new strategy of liver regeneration therapy for decompensated liver cirrhosis patients.

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HUMAN ADIPOSE TISSUE-DERIVED MULTILINEAGE PROGENITOR CELLS EXPOSED TO OXIDATIVE STRESS INDUCE NEURITE OUTGROWTH IN PC12 CELLS THROUGH P38 MAPK SIGNALING

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Mesenchymal stem cells (MSCs) are multipotent stem cells that can differentiate into various types of cells. These cells have been isolated from bone marrow, umbilical cord blood, and adipose tissue, and can be easily obtained and expanded *ex vivo* under appropriate culture conditions. Thus, MSCs are attractive materials for cell therapy and tissue engineering. Especially, human adipose tissue-derived mesenchymal stem cells, also referred to as human adipose tissue-derived multilineage progenitor cells (hADMPCs), have great advantages because of easier and safer access to adipose tissue obtained from lipoaspirates without serious ethical issues. Moreover, hADMPCs have more multipotent properties for regenerative medical applications than other stem cells. These cells have been reported to have the ability to migrate into the injured area, and differentiate into hepatocytes, cardiomyoblasts, pancreatic cells, and neuronal cell lineages. In addition, it is known that hADMPCs secrete wide variety of cytokines and growth factors for tissue regeneration. Recently, several groups have reported that hADMPCs facilitate neurological recovery in experimental models of stroke, and Parkinson's disease, but despite the superiority of hADMPCs over other stem cells, the potential use of hADMPCs for the treatment of these neurodegenerative disorders has not been fully investigated. Generally, damaged tissues, such as brain of patient suffered from ischemic stroke, are subject to inflammation, during which will cause generation of reactive oxygen species

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(ROS). Numerous studies have examined the crucial roles of oxidative stress in neurodegenerative disorders; e.g., stroke, Alzheimer's disease, Parkinson's disease. Thus, the influence of environmental stress in the lesions, including oxidative stress, on hADMPCs is important issue to be determined. In this study, we examined the roles of oxidative stress for hADMPCs in the neurite outgrowth in rat pheochromocytoma cell line PC12 cells. By treatment of buthionine sulfoximine (BSO), an inhibitor of rate limiting enzyme in the synthesis of glutathione, hADMPCs accumulated ROS, which resulted in the promotion of neurite outgrowth in PC12 cells. As activation of Smad1/5/8 and Erk1/2 MAPK was observed in PC12 cells, we further tried to identify the molecules that were secreted from hADMPCs, and found that bone morphogenetic proteins 2 (BMP2) and fibroblast growth factor 2 (FGF2) transcripts and secretions were increased in hADMPCs. Addition of N-acetylcysteine, a precursor of antioxidant intracellular glutathione, suppressed the BSO-mediated upregulation of BMP2 and FGF2, demonstrating that expression of these genes was regulated by oxidative stress. Moreover, BSO treatment caused phosphorylation of p38 MAPK in hADMPCs. Inhibition of p38 MAPK by pharmacological inhibitor SB203580 was sufficient to suppress BMP2 and FGF2 expression, while their expression was significantly upregulated by overexpression of constitutive active form of MKK3 and MKK6, which are upstream molecules of p38 MAPK. These results clearly suggest that glutathione depletion, followed by accumulation of ROS, stimulates the activation of p38 MAPK, which leads to neurotrophic factors, BMP2 and FGF2 expression in hADMPCs. Oxidative modification of BMP2 and FGF2 expression from hADMPCs is easy and safety way and may be applied to a new cell therapy for neurodegenerative disorders such as stroke and Parkinson's disease.

Poster Board Number: F-2296

A LOOK AT THE MECHANISM UNDERLYING HUMAN FETAL MESENCHYMAL STEM CELL HOMING

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The ability of mesenchymal stem cells (MSCs) to migrate to sites of injury and inflammation has made it an attractive candidate for tissue repair in the field of regenerative medicine. Studies have shown that MSCs have only one known adhesive pathway and a limited repertoire of chemokine receptors on the cell surface. However, how MSCs mobilize and migrate to the injury sites remain poorly defined. We hypothesize that human fetal MSCs (hfMSCs) can obtain cues from the inflammatory cells, specifically monocytes, in order to home to injury sites. Consistent with current literature, our data show that interactions of hfMSCs with human umbilical vein endothelial cells (HUVEC), under both static and flow conditions, is dependent on interactions between vascular cell adhesion molecule-1 (VCAM-1) and very late antigen-4 (VLA-4) expressed on HUVEC and hfMSCs respectively. In an attempt to enhance these interactions, hfMSCs were activated by tissue necrosis factor-α (TNF-α). This resulted in an increase in VCAM-1 and intercellular adhesion molecule-1 (ICAM-1) expression on the hfMSCs as assessed by flow cytometry. Instead of the expected enhancement, however, there was near complete abrogation of hfMSC-HUVEC interactions. This inhibition in hfMSC-HUVEC interactions correlated with a reduction in α4 integrin subunit expression, resulting in the inability of hfMSCs to tether onto HUVEC surface under flow conditions. In contrast, the binding of hfMSCs to HUVEC under static conditions was not affected by TNF-α treatment. When TNF-α activated-hfMSCs were co-perfused with myelomonocytic cell lines, HL60 and THP-1, there was a restoration of TNF-α activated-hfMSC-HUVEC interactions. Live-time imaging shows that hfMSCs utilize arrested leukocytes as 'bridges' to facilitate their arrest on endothelial cells. Analysis shows a 2.4 and 2.16 fold increase in the presence of HL60 and THP-1 respectively as compared to untreated hfMSCs at shear stress of 0.5 dynes/cm2. Treatment of TNF-α activated-hfMSCs with a functional blocking anti-VCAM-1 antibody resulted a 8.8 fold decrease ($p \leq 0.05$) in number of interacting cells compared to untreated control cells. Cells treated with a non-blocking antibody behaved similarly to the untreated control. This indicates that hfMSC recruitment is mediated predominantly by VCAM-1 expression on the hfMSCs. These findings support our hypothesis that hfMSCs utilize inflammatory cells, particularly monocytes, to target and localize to sites of tissue injury. Understanding the mechanisms behind MSC migration will allow us to enhance the number of MSCs targeting the specific tissues with subsequent acceleration in tissue healing.

Poster Board Number: F-2297

INTRAVENOUSLY-INFUSED HUMAN BONE MARROW-DERIVED MESENCHYMAL STEM CELLS PROMOTE SURVIVAL OF CORNEAL ALLOGRAFT BY REDUCING THE EARLY SURGERY-INDUCED INFLAMMATION.

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Mesenchymal stem/progenitor cells (MSCs) were reported to enhance the survival of cellular and organ transplants. However, their mode of action was not established. We here demonstrated that peri-transplant intravenous (IV) infusion of human MSCs (hMSCs) decreased the early surgically-induced inflammation and reduced the activation of dendritic cells in the cornea in a mouse model of corneal allotransplantation. Subsequently, immune rejection was decreased, and allograft survival was prolonged. IV hMSCs decreased the expression of chemokine (C-C motif) receptor 7, a key molecule for dendritic cell migration from the inflamed cornea to draining lymph nodes. Quantitative assays for human GAPDH revealed that <10 hMSCs out of $1x10^6$ cells were recovered in the cornea 10 hours to 28 days after IV infusion. Most of hMSCs were trapped in lungs where they were activated to increase expression of the gene for the anti-inflammatory protein TNF-α stimulated gene/protein 6 (TSG-6) upto 114-fold. IV hMSCs with a knockdown of TSG-6 did not suppress the early inflammation and failed to prolong the allograft survival. Also, IV infusion of recombinant TSG-6 reproduced the effects of hMSCs in reducing the early inflammation in allografts. Results suggest that hMSCs improve engraftment of corneal allografts by reducing the early surgery-induced inflammation.

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EFFECTS OF HUMAN DENTAL STEM CELL CONDITIONED MEDIUM ON THE OXIDATIVE STRESS-INDUCED CELL DAMAGE IN MC3T3-E1 PREOSTEOBLASTIC CELLS

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PURPOSE: The therapeutic effects of stem cell transplantation can be mediated by paracrine factors. To determine whether dental pulp stem cell conditioned medium (DPSC-CM) prevents the highly reducing sugar 2-deoxy-d-ribose-induced oxidative cell damage and stimulates the differentiation capacity of MC3T3-E1 preosteoblastic cells. METHODS: Primary cultures of DPSCs were established from explants of the human dental pulp. MC3T3-E1 preosteoblastic cells were cultured in alpha-MEM containing DPSC-CM for the experimental group and alpha-MEM alone for the control group in the presence of 2-deoxy-d-ribose. Time- and dose- dependent cell viability was monitored by CCK-8 assay, and the induction of apoptosis was demonstrated by the cell death ELISA kit. The intracellular oxidative stress was measured by fluorometric analysis of DCFH oxidation using 2′ ,7′ -dichlorofluorescin diacetate (DCFH-DA) as probe. Growth factors protein array, ELISA analysis and quantitative real time polymerase chain reaction (RT-PCR) were used to identify soluble mediates in DPSC-CM and osteoblastic differentiation markers. RESULTS: The highly reducing sugar, 2-deoxy-d-ribose decreased the viability of MC3T3-E1 preosteoblastic cells and caused concomitant increase in apoptotic cell death and increased the production of ROS. DPSC-CM suppressed the prooxidative effects of 2-deoxy-d-ribose, indicating involvements of antioxidant signaling in the mechanism. We found that DPSCs release soluble mediators into the culture medium, including HGF, EGF, FGF-4, IGF-1, IGFBP-1, IGFBP-2, IGFBP-4, IGFBP-6, IGF-2, TGF, VEGF, which are higher than that of bone marrow-derived mesenchymal stem cell conditioned medium (BMMSC-CM). In addition, treatment with DPSC-CM resulted in a significant elevation of ALP activity, collagen content, and mineralization in the cells. DPSC-CM increased the gene expression of differentiation, including ALP, collagen, AML, osteocalcin, OPG, OPN, FGF2, TGF-beta, BMP4 in MC3T3-E1 preosteoblastic cells. CONCLUSIONS: We have demonstrated the benefits of the conditioned medium derived from human dental pulp stem cell(DPSC-CM) in survival and function of MC3T3-E1 preosteoblastic cells following oxidative stress-induced cell damage.

Poster Board Number: F-2299

THE ROLE OF HUMAN BONE MARROW MESENCHYMAL STEM CELLS IN BASEMENT MEMBRANE FORMATION OF SKIN WOUND HEALING

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Bone marrow derived mesenchymal stem cells (MSCs) are widely recognized for their abilities to stimulate and accelerate wound healing. Recently, intradermal administration of allogenic MSCs have also been shown in human subjects to help to restore collagen VII deficit in the dermo-epidermal junction (DEJ) in patients with recessive dystrophic epidermolysis bullosa. Interestingly, efficacious treatment with MSCs required lower cell numbers in comparison to fibroblast cell therapy. This suggests that MSCs might have a greater potential in regenerating the DEJ. The mechanism and the required signals by which MSCs contribute to basement membrane formation however remains largely unknown. In our experiments, MSCs were compared with human dermal fibroblasts (HDF) for their matrix deposition properties and patterns both alone and when co-cultured with the *in vitro* spontaneously transformed normal human keratinocyte cell line, HaCaT. Emphasis was placed on the deposition of DEJ proteins such type IV and VII collagen and laminin 5 (laminin 3,3,2). Type I collagen as the dermal partner for anchoring fibril was also studied. Here, we applied macromolecular crowding (MMC) as a tool to facilitate the *in vitro* deposition of matrix proteins. MMC allowed us to observe the deposition of extracellular matrix and its organization in a two dimensional *in vitro* system. Immunocytochemistry showed that MSCs deposit across the board more matrix per cell than HDF. In co-culture, HaCaTs and MSCs segregated clearly with defined boundaries between cell types. Similar features were observed with HaCaT/HDF, but with the boundaries were less defined. The extracellular matrix deposition pattern also reflected the physical organization of the cells. Laminin 5 and type VII collagen were deposited more specifically at the boundaries between HaCaTs and MSCs. The deposited basement membrane proteins formed a clear border between them, reminiscent of a basement membrane zone. The same deposition pattern however was not observed in keratinocyte-fibroblast co-cultures: while there was an increased deposition of laminin 5 and type VII collagen at the region around the cell boundaries, no defined borders were identified. It was then observed that type VII collagen expression in MSCs monocultures were increased in samples with MMC and ascorbic acid. The deposited collagen VII had a granular structure and appears to co-localize with type I collagen. Similar deposition was observed in HDFs but with less intensity. In the absence of ascorbic acid, type VII collagen expression in co-cultures appeared to be restricted to the HaCaT population which agrees with published data. However, when ascorbic acid was added to facilitate the extracellular secretion of collagen and its deposition enabled by the use of MMC, we saw that type VII collagen staining was stronger in the MSC/HDF population region and again, appeared co-localized with type I collagen. The expression of type VII collagen was also increased when TGF-β1 was added to the MSC and HDF monocultures. Our current data suggest that MSCs may be responsible for the secretion and maybe organization of extracellular matrix and basement membrane in recovering skin wounds. Further investigations are on-going to better understand how MSCs may affect matrix deposition and organization in order to reveal the mechanism through which MSCs facilitate wound healing and basement membrane formation.

Poster Board Number: F-2300

THE ROLE OF HIF1A IN REGULATION OF TELOMERASE LEVELS IN MOUSE ADULT STEM CELLS

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Telomerase is vital to ensure the long term self-renewal capacity of stem cells. The primary mechanism which limits telomerase in mammals is via the suppressed expression of the catalytic component of telomerase, telomerase reverse transcriptase (Tert). Previously we have identified hypoxia inducible factor (HIF1α) as a transactivator of Tert in mES cells. In the present study, we have begun to assess the potential role of HIF1α in regulating telom-

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erase and telomere length in adult somatic stem cells, including hematopoietic stem cells (HSC), neuronal stem cells (NSC), and others. We find low but detectable levels of HIF1α in murine HSC (cKit+Sca1+Thy1.1LoLinNeg), and show that transient culture (48 hours) of these cells in hypoxic conditions (1% oxygen) is sufficient to enhance both HIF1α levels as well as telomerase activity and Tert expression. Furthermore, immunohistochemical analysis of mouse brain sections revealed the presence of HIF1α positive cells in neural stem cell niches, the subgranular zone of dentate gyrus in hippocampus and subventricular zone of the lateral ventricles, co-localizing with the NSC marker CD133. Our results suggest that HIF1α may also play an important role in regulating telomerase levels in adult stem cells, in addition to ES cells.

Poster Board Number: F-2301

HYPERBARIC OXYGEN STIMULATES ADIPOSE DERIVED MESENCHYMAL STEM CELLS GROWTH AND DIFFERENTIATION IN STREPTOZOCIN INDUCED DIABETES MELLITUS TYPE 1 MICE *IN VIVO*

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Hyperbaric oxygen modifies function of stem/progenitor cell (SPCs) and enhances new blood vessels formation. In wounds healing, VEGF secreted from adipose-derived mesenchymal stem cells (ASCs) induces migration and proliferation of endothelial cells, increasing the vascularity of a wound bed. We hypothesized that oxidative stress from hyperbaric oxygen (HBO_2, 2.8 ATA for 90 min daily) would exert a trophic effect on adipose- derived mesenchymal stem cells in normal mice and in a streptozocin-induced diabetes mellitus mouse model via reactive oxygen /reactive nitrogen species (ROS/RNS)-dependent mechanisms. Adipose-derived stem/progenitor cells (ASCs:Sca-1+/CD31-/DAPI-), were sorted from omentum of normal mice, labeled with the fluorescent dye 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE), added to Matrigel and administered subcutaneously, and the mouse then subjected to HBO_2 treatment. In combination, HBO_2 and ASCs demonstrated cumulative effects. Vascular channels lined by $CD34^+$ SPCs were identified and they were significantly reduced in diabetic animals. Matrigel with seeded ASCs after HBO_2 exhibited accelerated channel development, cell differentiation based on surface marker expression and cell cycle entry. Blood-borne and bone marrow $CD34^+$ SPCs of diabetic animals versus normal mice had lower amounts of thioredoxin-1 (Trx1), Trx reductase (Trx1R), hypoxia-inducible factors (HIF)-1, -2, and -3, phosphorylated mitogen-activated protein kinases (pMAPKs), vascular endothelial growth factor (VEGF) and stromal cell-derived factor-1. We conclude that thioredoxin system activation leads to elevations in HIF-1 and -2, followed by synthesis of HIF-dependent growth factors. HIF-3 has a negative impact on SPCs. By causing an oxidative stress, HBO_2 activates a physiological redox-active autocrine loop in recruited SPCs and increased paracrine secretion in seeded ASCs, resulted in the stimulated neovasculogenesis.

Poster Board Number: F-2302

NEUROPROTECTIVE EFFECT OF FTY720 AND SEW2871 ON H2O2 INDUCED ALZHEIMER *IN VITRO* MODEL OF DIFFERENTIATED RAT PRIMARY HIPPOCAMPAL STEM CELLS.

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Alzheimer's disease (AD) is the most common form of dementia and irreversible, progressive brain disease that slowly destroys memory and thinking skills. There is no cure for the disease, which worsens as it progresses, and eventually leads to death. The damage spreads to a nearby structure in the brain called the hippocampus, which is essential in forming memories. Scientists don't yet fully understand what causes AD, but it has become increasingly clear that it develops because of a complex series of events that take place in the brain over a long period of time by Age-related changes include atrophy (shrinking) of certain parts of the brain, inflammation, the production of unstable molecules called free radicals, and mitochondrial dysfunction. It is meanwhile well described, that neuronal loss in case of AD is due to various types of stress conditions. These comprise oxidative stress, excitotoxicity, Ca++-overload and others beyond amyloid toxicity. Evidence has been accumulating that free radicals and reactive oxygen species (ROS) have been associated with the etiology and progression of different neurodegenerative diseases. Free radicals play an important role in the development of neurodegenerative disorders such as Alzheimer's disease (AD), Huntington's disease (HD), Pick's disease, amyotrophic lateral sclerosis, epilepsy, schizophrenia, cerebral ischemia. Free radical generation leads to oxidative damage of lipids, nucleic acids and proteins, abnormal aggregation of cytoskeletal proteins, mitochondrial dysfunction, antioxidant enzymes upregulation, reactive nitrogen species formation, advanced glycation end products formation, inactivation of key enzymes. Primary hippocampal Neurons can be cultured and exposed to the H2O2 as an induced cell inflammation and toxicity Cell death for modeling of AD in a controlled *in vitro* area for evaluation of using neuroprotective agents. Model systems such as experimental animals and cell cultures are often used to understand how oxidative stress can produce neurotoxic effects, which later lead to neuronal dysfunction, degeneration, and cell death. This study investigated whether FTY720 and SEW2871 was able to extend their unique neuroprotection to primary cultured differentiated rat hippocampal neural stem cells against hydrogen peroxide induced inflammation cell damage.

Poster Board Number: F-2303

THE ROLES OF BURR-HOLE AND CALVARIAL BONE MARROW STEM CELL IN THE ISCHEMIC RAT BRAIN MODEL

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Moyamoya disease is a cerebrovascular disorder characterized by chronic progressive stenosis of the intracranial internal carotid arteries (ICAs), including the proximal anterior cerebral arteries (ACAs) and middle cerebral arteries (MCAs). Current treatments are designed to prevent strokes by improving blood flow to the affected cerebral hemisphere, not to reverse the primary

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disease process. Two general surgical methods are used: direct and indirect revascularization. The burr-hole method, one of the indirect methods, is reported to be very simple and effective for revascularizing the territory of the anterior cerebral arteries (ACA) not only in children but also in adults with definite moyamoya disease. However, the treatment mechanism of this method has not been explained, yet. Recently there were some reports which provide clues for explaining it. Angiogenesis of the lower extremities could be improved by multiple burr-holes on tibia via autologous bone marrow stem cell transplantation in Beurger's disease. The transplantation of autologous whole BMCs by fenestration of the tibia bone represents a simple, safe, and effective means of inducing therapeutic angiogenesis in patients with Buerger's disease. Total avulsion injury of scalp was successfully treated with multiple burr-holes and skin graft. The purpose of this study is to investigate the role of burr-hole and bone marrow stem cells in ischemic rat brain mode and the treatment mechanism of multiple burr-hole technique in moyamoya disease. Twenty SD rats (250 g) were divided into four groups: normal group (5), burr-hole group (5), focal ischemia group (5), burr-hole + focal ischemia group (5), respectively. Focal ischemia was induced by transient middle cerebral artery occlusion (MCAO). At 2 weeks after focal ischemia, bilateral burr-holes were made at the parietal bone with minimal cortical incision. Bromodeoxyuridine (BrdU, 50 mg/Kg) was injected intraperitoneally, 2 times a day for 6 days. At 1 week after burr-hole making, brain was harvested and coronal sections around the burr hole were obtained. Immunohistochemical analysis included BrdU, VEGF (Vascular Endothelial Growth Factor), CD34 (mesenchymal stem cell marker), doublecortin (immature neural marker), and Nestin (neural stem cell marker). In burr-hole + focal ischemia group, BrdU (+), CD34 (+) cells at the cortical incision site beneath the burr-hole showed infiltrative pattern into the surrounding brain parenchyme. VEGF were increased around the burr-hole site. These cells also were positive for doublecortin and nestin, which means bone marrow stem cells (mesenchymal stem cell) transformed into neural precursor cells or other precursor cells. In conclusion, data in these studies indicated that calvarial bone marrow stem cells around the burr-hole play a role in angiogenesis and neurogenesis in ischemic brain injury. Also, these can do an important role in the multiple burr-hole technique of moyamoya disease.

Poster Board Number: F-2304

AUTOLOGOUS MESENCHYMAL STEM CELLS IMPROVE SKELETAL MUSCLE FUNCTION AFTER SEVERE INJURY IN BOTH SEXES

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Mesenchymal stem cell (MSC) therapy has been shown to have the potential to enhance muscular regeneration. In previous studies our group was able to show a dose-response relationship in female animals between the amount of transplanted cells and muscle force. The impact of sex on the regeneration of musculoskeletal injuries following MSC transplantation (TX) remains unclear. 36 SD-rats received an open crush trauma of the left soleus muscle. One week after trauma 2.5 x 10^6 autologous MSCs, harvested from tibial biopsies, were transplanted locally (female n=9, male n=9). Control animals received saline solution (female n=9, male n=9). Histologic analysis and biomechanical evaluation by in-vivo contraction force measurement were performed 3 weeks after TX. MSC-TX improved the force of the injured soleus muscles in males significantly (twitch: treated 0.76 (0.51 - 1.15), untreated 0.45 (0.32 - 0.73, p=0.01; tetany: treated 0.63 (0.4 - 1.21), untreated 0.34 (0.16 - 0.48), p=0.04). Force measurements in females also showed significant improvements (twitch: treated 0.71 (0.38 - 0.96), untreated 0.36 (0.18 - 0.63), p=0.005; tetany: treated 0.53 (0.21 - 0.68), untreated 0.27 (0.11 - 0.47), p=0.01). The intersexual comparison of fast twitch and tetanic contraction forces revealed no significance (twitch, p=0.55, tetany, p=0.19). The histological analysis showed no differences in the amount of fibrotic tissue (male p=0.9, female p=0.14) and the size of muscle area (male p=0.2, female p=0.56) following treatment. Male animals showed higher values for muscle area (male vs. female p=0.011) and less fibrosis (male vs. female p=0.028) independent of treatment. The regeneration potential of male and female individuals has been the subject of academic controversy. The present study demonstrates that the outcome of skeletal muscle regeneration after injury can be improved in both sexes via the transplantation of MSC.

Poster Board Number: F-2305

ROLES OF ADAM8 FOR SKELETAL MUSCLE REGENERATION

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During skeletal muscle regeneration, activated myoblasts replace damaged muscle tissues with newly-formed muscle fibers. In addition to skeletal muscle lineage cells, inflammatory cells including macrophages are involved in its regeneration. Macrophages remove damaged cells and also promote tissue remodeling by activating myogenesis or regulating angiogenesis. Proteases can contribute to skeletal muscle regeneration by proteolytic shedding of inflammatory cytokines, receptors, and cell adhesion molecules (Edwards et al., Mol Aspects Med., 2008). ADAM8 (a disintegrin and metalloprotease 8) is a membrane-bound metalloprotease identified in macrophages originally. We previously showed that ADAM8 is involved in the onset of primitive blood circulation in zebrafish. ADAM8 expressed in erythroblasts abrogates blood-to-vessel adhesion via ectodomain shedding of cell-adhesion molecules (Iida et al., Curr Biol., 2010). In contrast, *ADAM8*$^{-/-}$ embryos have no obvious developmental defect (Kelly et al., Dev Dyn., 2005), however roles of ADAM8 in adult mice are still unclear. Because ADAM8 expression is elevated under the inflammatory conditions, we hypothesized ADAM8 might play roles in skeletal muscle regeneration. To determine whether ADAM8 contributes to skeletal muscle regeneration, we used two types of muscle regeneration models: cardiotoxin (CTX) induced skeletal muscle injury and dystrophin-*null* mice, models for Duchenne muscular dystrophy. We confirmed increased expression of ADAM8 upon induction of skeletal muscle regeneration. To induce acute skeletal muscle injury, CTX was injected into the tibialis anterior muscle (TA) of wild type and *ADAM8*$^{-/-}$ mice. Although skeletal muscle regeneration occurred in the latter mice, their muscle fibers were thinner than those of wild type mice, suggesting delayed or insufficient skeletal muscle regeneration in the ADAM8$^{-/-}$ mice. To examine whether ADAM8 affects macrophage functions, sections of TA were immnostained with F4/80 as a marker of macrophage. At 7 days after skeletal muscle injury, macrophages were present in both of wild type and *ADAM8*$^{-/-}$ mice. However, in the *ADAM8*$^{-/-}$ mice, large clusters of macrophages were frequently observed in degraded skeletal muscle fibers. Next, we generated dystrophin-*null*; *ADAM8*$^{-/-}$ mice. The dystrophin-*null*; *ADAM8*$^{-/-}$ mice had massive infiltrated immune cells positive

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for F4/80 and CD11b. These results suggest that dispersion or clearance of macrophages during skeletal muscle regeneration is affected in *ADAM8*$^{-/-}$ mice.

Poster Board Number: F-2306

MOLECULAR BASIS OF STEM CELL DYNAMICS IN PLANARIAN REGENERATION

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Planarians can regenerate a whole individual from tiny tissue fragments from almost any part of their bodies based on well-characterized somatic pluripotent stem cells called neoblasts, which can self-renew and give rise to all missing cell types including brain cells. Neoblasts are morphologically defined as a unique population of cells and specifically express a set of stemness genes. We recently identified MAPK signaling pathways that play crucial roles in the regulation of neoblast dynamics during regeneration of the planarian Dugesia japonica. JNK signaling is required for active mitosis of undifferentiated neoblasts and generation of blastema cells, whereas ERK signaling is required for differentiation of neoblasts into multiple cell types, including the brain in the head region, gut and pharynx-forming muscle cells at the middle portion of the body. In the process of neoblast differentiation during regeneration, they must recognize their correct location along the anterior-posterior axis to develop into appropriate tissues and organs. Here, we examined during head regeneration from tail fragments and identified a fibroblast growth factor receptor-like gene nou-darake as a head blastemal component and ß-catenin signaling that is required for tail identity, both of which are involved in the modulation of ERK signaling and provide appropriate positional information to neoblasts during regeneration.

Poster Board Number: F-2307

EPIGENETIC IMPACTS OF ACYL-L-CARNITINES IN RELATION TO SELECTED CONGENITAL DISORDERS

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Acetylcarnitine esters (LCs) are formed intracellularly during regular metabolic activity. LCs transport fatty acyl moieties into the mitochondria. Acetyl-L-carnitine (ALC) is the principal acylcarnitine ester. ALC participates in both anabolic and catabolic pathways in cellular metabolism and interacts in membrane and molecular levels. This study delineates the epigenetic mechanism of LCs in relation to selected congenital disorders. LC could help cells to recover from DNA SSBs induced by different types of DNA lesions and the use of LC as a protection against oxygen free radicals (OFRs). It was reported that p53 facilitates differentiation by translocating to the nucleus and associating with the Nanog promoter and inhibiting its transcription, suggesting that the role of p53 is important during differentiation than in responding to DNA damage in stem cells, and it has been documented that ALCs enhances HSP-70 and p53 gene expressions in a variety of inflammatory conditions including congenital disorders, aging and neoplasms. The intracellular concentration of free CoA and acetyl-CoA is recognised to be a common mechanism for the various physiological activities of ALC, such as the acetylation of H4 histones and prevents telomere attrition. ALCs augments the repairing enzyme poly(ADP-ribose)polymerase in acute oxidative stress. L-carnitine, inhibits cytogenetic expression of the fragile-X site in cultured lymphocytes of patients, suggesting that the interaction of these substances with the chromatin structure at the fragile site was present. ALC reduces levels of MDA, 8-HDG, quenches neutrophil superoxide and nitric oxide radicals, IL-10 and IL-2 gene expressions, cytosolic cytochrome-c and caspase-3 active fragments expression in xenobiotic induced oxidative stress. Studies of patients with both hypothyroidism and hyperthyroidism show decreased levels of L-carnitine (LC) in muscle cells. LC blocks the entry of excess thyroid hormone into the cell nucleus of liver cells, neurons, and cardiac cells, thereby reducing the symptoms of hyperthyroidism. ALCs augments the expression of urea cycle enzymes in patients suffering from juvenile visceral steatosis (JVS). LC is beneficial in prevention of oxidative stress and augmentation of ETC complex I in discrete regions of brain associated with Parkinson's disease. ALC exerts free radical scavenging effects, restores redox status, prevents β-amyloid accumulation and iron-chelation and beneficial in the management of Alzheimer's disease (AD). LC is beneficial in the management of epileptic patients on valproic acid. ALC is beneficial in the patients suffering from Down's syndrome (DS), Multiple Sclerosis (MS), Huntington's disease (HD), Muscular dystrophy (MD), Phenylketone uria (PKU) and Pyronie disese (PD). ALC is beneficial in senile dementia and age related impairment of cognitive functions and mood disorders and depression and hence ALCs are potent epigenetic molecular medicines to be considered in cryobiology, stem cell research, anti-aging and regenerative medicine.

Poster Board Number: F-2309

COMPARISON OF THE EFFECTS OF THREE MESENCHYMAL STEM CELLS- CONDITIONED MEDIUMS IN WOUND HEALING

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Keeping skin integrity has special value. Mesenchymal stem cells are useful in wound healing and skin repair. Recently, mesenchymal stem cells-conditioned mediums are discovered to have almost the same beneficiary effect on skin, but conditioned medium of which source has the best effect remained to be investigated. The purpose of this study was to compare the effect of conditioned medium of three sources of mesenchymal stem cells in wound healing. Fibroblasts were treated with adipose derived stem cell-conditioned medium (ADSC-CM), bone marrow mesenchymal stem cell-conditioned medium (BMSC-CM) and umbilical cord blood stem cell-conditioned medium (UCBSC-CM) and DMEM/F12 with 10% FBS as a control group. Gene expressions of hyaluronan (HA), collagen type I, fibronectin and secreted amount of HA, fibronectin and collagen I were measured. Fibroblast migration and proliferation were also assessed. The difference of some gene expressions between the treated groups and the control were significant but they were not very obvious .The secreted amount of HA, fibronectin and collagen was at highest point in the group of fibroblasts treated with ADSC-CM. Fibroblast migration rate was at the fastest point in the control group containing 10% FBS and then in the group treated with UCBSC-CM. No significant difference between the proliferations of 4 groups was observed. Among mesenchymal stem cells, ADSC have more stimulatory effect on fibroblasts to secret more ECM components and that could be due to the difference of amount or type of secreted growth factors and cytokines from it.

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Poster Board Number: F-2310

THE ROLE OF INFLAMMATION IN ADULT CARDIOMYOCYTE REPLENISHMENT AFTER MYOCARDIAL INFARCTION

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Following myocardial infarction, cyclooxygenase-2 (COX-2) signaling pathway is activated as part of the inflammatory responses and inhibition of COX-2 contributes to heart failure, suggesting its role in cardiac protection. Using a genetic fate-mapping approach, we discovered that administration of the prostaglandin E2 (PGE2), a COX-2 downstream product, could evidently restore cardiomyocyte replenishment in aged mice (>18 months). Quantification of stem cell marker expression revealed that Sca-1 level was induced following PGE2 treatment. Moreover, the number of total Sca-1+ cells and the cells co-expressing mature cardiomyocyte maker cardiac troponin T (cTnT) also increased at the infarcted region of the aged heart. As PGE2 is an inflammation modulator, we then investigated the interaction between inflammatory cells and cardiomyocyte replenishment in young mice. We screened several markers representing different types of inflammatory cells include CD3 for T cell, B220 for B cell and CD11b for myeloid cells. Among all markers analyzed, only CD11b expression level elevated in response to PGE2 treatment in both young and aged mice. Macrophage is one of CD11b+ cell types has been shown to be related to tissue regeneration. Therefore, we examined which specific macrophage subtype, M1 or M2, is involved in the *de novo* cardiomyocyte formation. Immunostaining revealed that the number of CD68+/CD206+ M2 macrophage increased after PGE2 treatment, while fewer CD11b+/CD11c+ M1 macrophages were observed in the infarcted heart upon PGE2 treatment. Furthermore, gene expression level of anti-inflammatory cytokine IL-10, produced by the M2 macrophage, was elevated in response to PGE2 treatment in both young and aged mice. Finally, we observed that PGE2 significantly reduced the expression of aging-associated markers including TGF-β1, p53 and p19 in aged mice. Taken together, these findings indicate that PGE2 may modulate the post-infarction inflammatory micro-environment, possibly through changing the M1/M2 macrophage ratio, to restore cardiomyocyte replenishment in aged mice.

Poster Withdrawn

Other

Poster Board Number: F-2312

VERY SMALL HUMAN OCT-4+/CD45-/LIN-/CD133+ STEM CELLS DERIVED FORM UMBILICAL CORD BLOOD CONSIST OF DISTINCT PLURIPOTENT SUBPOPULATIONS

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Very small embryonic-like stem cells (VSELs) derived from human umbilical cord blood (UCB) are i) very small (<6 μm), ii) rare, iii) non-hematopoietic CD45-/Hematopoietic lineages' markers (Lin)- cells which may iv) express several stem cell markers such as CD133, CD34 or CXCR4 and v) contain the highest number of Oct-4+ cells in CD45-/Lin-/CD133+ subpopulation. Similar, murine adult bone marrow- derived VSEL counterpart that has been well characterized, represents quiescent and diploid very small stem cells that may give rise into cells from all three germ layers as well as play beneficial role in regeneration of injured organs following transplantation. However, our understanding of features and physiology of human UCB- derived VSELs are still challenging. Thus, in current study we examined distinct subpopulatins of UCB- derived VSELs in terms of co-expression of several stem cell antigens including CD133, CD34 and CXCR4 as well as features released to their stemness such as quiescence and telomerase activity. Our flow cytometric data indicate that 53.33±20.13% and only 2.48±0.12% of human CD45-/Lin-/CD133+ VSELs co-express CD34 and CXCR4 antigens, respectively. Importantly, we established significant differences in terms of the expression of pluripotent genes such as Oct-4, Nanog, Dppa1, Sox2, between purified CD45-/Lin-/CD133+ subfractions of cells with and without expression of both vast antigens. Despite the fact of very small size of CD45-/Lin-/CD133+ VSELs requiring modified cell sorting strategies, based on our cell cycle and DNA content analysis, we established that human they are normal diploid and quiescent non-proliferating cells (93.95±2.05%), similarly to UCB hematopoietic stem cells and mature lymphocytes. Moreover, they exhibit high telomerase activity. In conclusion, we established that UCB contains rare population of very small in size and Oct-4+/CD45-/Lin-/CD133+ pluripotent cells with different co-expression of important markers such as CD133, CD34 and CXCR4. These

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newly identified subpopulations may possess different characteristics of pluripotency as well as differentiation potential. Taking into consideration that VSELs may be potentially employed for regenerative purposes, importance of these diversity need to be further characterized.

Poster Board Number: F-2313

IDENTIFICATION OF VERY SMALL PLURIPOTENT OCT-4+/NANOG+/VCAM+/CD45-/LIN- STEM CELLS IN ADULT RAT BONE MARROW

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Our previous studies have shown that both adult mouse and human bone marrow (BM) tissue may harbor a rare population of adult stem cells with pluripotent characteristics (Leukemia 2006; 20:857-69; Eur J Haematol 2010;84:34-46). These unique very small embryonic-like stem cells (VSELs)adult stem cells were found as i) very rare cells (less than 0.001% of total cells), ii) smaller than erythrocytes (less than 4 and 7 microns in diameter, in mice and humans, respectively) which iii) do not express CD45 and markers of mature hematopoietic lineages (Lin), but iv) do express CD133, CD34 or CXCR4 markers (in humans) and Sca-1 antigen (in mice). Despite being non-adherent cell fraction, VSELs exhibit greater adhesion properties to synthetic and glass surfaces. Although, both murine and human VSELs have been described and their differentiation potential has been shown in vitro and *in vivo*, such cells in other mammals including rats have never been identified. Therefore, the aim of this study was to identify and characterize VSEL counterpart in adult rat BM that would represent very small stem cells CD45-/Lin- cells. To fulfill the goal, using flow cytometric technologies, we have screened several potential surface markers flagging rat BM-VSELs including predominantly multiple adhesion molecules. BM cells were isolated from both i) wild type and ii) transgenic eGFP-expressing adult Wistar rats and stained for rat CD45 antigen, hematopoietic Lin markers (including TCRαβ, CD3, CD11b, CD45RA) and selected selection markers including CD106. Rat BM-VSELs were identified and purified by multiparameter fluorescence- activated cell sorting (FACS) and subsequently the sorted fractions were examined for pluripotent markers expression such as Oct-4, Nanog, Rexo-1 on both mRNA and protein levels by employing real time RT-PCR and imaging cytometry (ImageStream system), respectively. We have established that pluripotent Oct-4+/Nanog+ stem cells may be identified in purified sorted fraction of small (FSC-low/SSC-low), CD45-/Lin- /CD106 (VCAM)+ cells which express 4.91±0.11, 4.37±1.33 and 3.78±1.20 times more of mRNA transcripts for Oct-4, Nanog and Rexo-1, respectively, when compared to unpurified rat BM cells. Such primitive Oct-4+/Nanog+ stem cells were also identified in the sorted fraction by ImageStream system analysis. In the current study, we identified and purified from rat adult BM a population of small cells resembling VSELs based on their 1) very small size (FSC-low/SSC-low), 2) negativity for CD45 and Lin markers, 3) expression of adhesion molecules such as VCAM and 4) expression of pluripotent markers including Oct-4 and Nanog on both mRNA and protein levels. Further examination of potential functional properties and applications of these cells in in vitro and *in vivo* models need to be performed.

Poster Board Number: F-2314

HUMAN INDUCED PLURIPOTENT STEM CELL BANKING FOR DRUG DISCOVERY RESEARCH

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Utilization of human embryonic stem (ES) cells as a tool for drug toxicity has been considered still problematic in Japan because of ethic issue and short supply though mouse ES cells are using for *in vitro* testing of drugs. Human induced pluripotent stem (iPS) cells offer an alternative. Then, we have embraced iPS cell technology to develop a drug screening system. Before iPS cells can be used in drug development, however, they need to be standardized and quality controlled that would enables pharmaceutical companies to do clinical study for the drug. In 2008, NIBIO has combined a 5-year Super Special Consortium to establish a drug testing system based on iPS cells. As part of the project, we are characterizing cell phenotypes of iPS cells using a Xeno-free defined culture medium and also developing protocols for producing endoderm for hepatocytes or neuroectoderm for neural cells from iPS cell lines. We expect that the protocols using the Xeno-free defined medium will be the foundation for human iPS cell research in pharmacological testing. Besides, as Japanese Collection of Research Bioresources (JCRB) cell bank, we have established the banking system including qualification methods, functional evaluation methods, and culture methods for iPS cells in order to supply standardized and quality controlled stocks to bank users. Seven lines of the qualified cells are now available from our bank.

Poster Board Number: F-2315

CRYOPRESERVATION OF HUMAN EMBRYONIC STEM CELLS WITH A MAGNETIC FIELD PROGRAMMED FREEZER

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Human embryonic stem cells (hESCs) have great potentials as a source of cells for tissue engineering and cell-based therapies since they have the ability to proliferate indefinitely and differentiate into all three germ layers. The obstacles need to overcome immediately are the cell survival rate and cell quality after cryopreservation. We know that when cells are frozen, the crystal of water molecule within the cells will grow up and damage the cell membrane. The purpose of this study is to increase the efficiency of colony formation and attachment of hESCs after cryopreservation by CAS (Cell Alive System), which is combining a programmed freezer with a magnetic field in freezing process. The hESCs were frozen by CAS until -32°C and transferred to -80°C refrigerator overnight and then stored in liquid nitrogen. After thawing, the cells were seeded on MEF in medium (w/ 10 μM Y-27632) for two days and exchange the medium (w/o Y-27632) afterward. The results of alkaline phosphatase staining showed that the CAS could significantly increase the efficiency of colony formation and attachment comparing with the Mr. Frosty, especially in lower DMSO concentration (5%). Besides we have confirmed that the cryopreserved cells could be subcultured, express pluripotent markers, and differentiate into three germ lay-

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ers. Here we demonstrate that the CAS is superior to Mr. Frosty for the cryopreservation of human embryonic stem cells.

Poster Board Number: F-2316

DIFFERENTIAL INVOLVEMENT OF IFN-GAMMA AND TNF-ALPHA IN IMMUNOSUPPRESSION MEDIATED BY HUMAN ADIPOSE-DERIVED MESENCHYMAL STEM CELLS

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The immunosuppressive activity of bone marrow mesenchymal stem cells (MSCs) has received considerable attention during the last few years, but immunosuppressive activity of human adipose-derived mesenchymal stem cells (hASCs) had been not studied in fine details. According to accumulated information, various molecular mechanisms can be involved in MSC-mediated immune suppression. These mechanisms underlying MSCs-mediated immunosupression include soluble factors such as pro-inflammatory cytokines IFN-γ, TNF-α, IL-1 and cell-cell contacts, which possibly depend on expression of cell adhesion molecules. To dissect some of the candidate mechanisms of MSC-dependent immune suppression *in vitro* co-culture system consisting of hASCs and phytohemaglutinin-activated peripheral blood lymphocytes (PBL) was established. According to our data hASCs inhibited proliferation of activated PBL both in trans-well and cell-contact co-culture system as assessed by CyQuant cell proliferation assay. hASC-conditioned and unconditioned growth medium had no effect on proliferation of activated PBL. Co-cultivation of hASCs and activated PBL at 1:100 ratio resulted in significant increase of both IFN-γ and TNF-α in growth medium according to the measurements of protein level of cytokines using 17-plex Bioplex cytokine kit. At the same time, concentrations of other cytokines varied slightly. Increment of IFN-γ and TNF-α was more than 1.5 fold in comparison to supernatants of separate cultures of hASCs and PBL. Elevated level of IFN-γ was detectable only under conditions of close contacts between hASCs and PBL cells. Meanwhile the level of TNF-α was found to be specifically increased following cell contact independent cultivation using semi-permeable membranes allowing for exchange of secreted factors between hASCs and PBL. Co-cultivation of hASCs and PBL at 1:10 ratio resulted in dramatic (at least 2 fold) drop of secreted IFN-γ and TNF-α levels independently of co-culture conditions (contact vs. contact free). Activation of lymphocytes with hASCs had also resulted in increased attachment of immune cells to hASCs. Attached cells were analyzed by flow cytometry following staining of cells with fluorophore-conjugated antibodies against CD4 and CD8 T cells, NK and B cells surface markers. It was found that proportion of activated CD4 T cells bound to hASCs increased significantly in comparison to non-activated. Meanwhile the proportion of other cell types in contact with hASCs decreased. Thus, we suggest that contacts between CD4 T cells and hASCs could possibly trigger secretion of IFN-γ by hASCs. Analysis of the population of activated CD4CD25 T cells revealed that proportion of these cells was reduced more than 2-fold when hASCs and PBL were cultured in transwells at a ratio of 1:10. In the same cultures, a proportion of apoptotic CD4CD25 T cells had been 2-10 folds lower than in control samples. The proportions of CD4CD25 T cells and apoptotic CD4CD25 T cells had not changed significantly under conditions of PBL to hASCs ratio 1:100 and in the presence of contacts between hASCs and PBL. Taken together our data support the substantial role of IFN-γ and TNF-α in hASC-mediated immune suppression and differential involvement of these cytokines in distinct mechanisms of hASCs activation.

Poster Board Number: F-2317

A PHASE I/II CLINICAL TRIAL TO ASSESS THE SAFETY AND EFFICACY OF BONE MARROW DERIVED ALLOGENEIC "HUMAN MESENCHYMAL STEM CELLS" IN PATIENTS WITH CRITICAL LIMB ISCHEMIA

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Stem cells are known for their ability to repopulate damaged organs to carry out the process of regeneration and repair. Adult multipotent mesenchymal stem or stromal cells (MSCs) have been at the forefront of basic and clinical research for decades. Several distinctly different biological properties have made these stem cells an attractive source for regeneration of tissues damaged by disease or injury. MSCs are known to differentiate into osteocytes, chondrocytes and adipocytes in vitro, and are also known to transdifferentiate into cells of other germ layers. MSCs possess unique characteristics that make these cells potentially useful for allogeneic therapy. MSCs home and accumulate in and around the damaged tissue to initiate the repair process through paracrine activity. Secondly, because of their non-immunogenic nature and potent immunosuppressive properties, these cells have the ability to survive in an allogeneic environment. Several types of angiogenic growth factors are secreted by MSCs both in vitro and *in vivo*, which suggest that these cells might be useful for treating diseases like critical limb ischemia (CLI), in which the natural process of angiogenesis of lower extremities has been severely impaired. Since BMMSCs have been the most well characterized population of MSC, with high regenerative potential, we have developed an investigational medical product (IMP) using pooled BMMSCs from normal adult healthy volunteers. The pooled MSC population has been extensively characterized for their expansion potential, surface marker expression, in vitro differentiation potential and also screened for angiogenic factor expression and secretion. Preclinical toxicity studies performed in rodent and non-rodent animals did not reveal any toxicity and the cells were found to be non tumorigenic in SCID mice. In addition, preclinical efficacy study performed with nude mice showed therapeutic efficacy of these cells. We have conducted a Phase I/II clinical trial, after receiving the approval from the Indian FDA, in no revascularization option patients afflicted with critical limb ischemia (CLI) to primarily determine the safety of intra muscular administration of allogeneic BMMSC with a single dose of 2.0+E06 per Kg body weight. Ten patients were enrolled in the cell therapy arm while 10 patients were treated with placebo. The trial was unblinded after six months and BMMSCs administration has been found to be safe. No immunological abnormalities have been observed with respect to blood lymphocyte subsets or with cytokine profile in both groups. In terms of efficacy, we observed statistically significant increase in ankle brachial pressure index (ABPI) and ankle pressure (AP) in BMMSC treated group of patients compared to the placebo. However, we did not observe significant difference between the two groups of patients in terms of rest pain and amputation free survival. Our results demonstrate that administration of allogeneic BMMSC is safe in CLI patients and we

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are currently conducting a Phase IIb dose ranging study in larger group of patients.

Poster Board Number: F-2318

ROLE OF MIR-335 IN THE IMMUNOREGULATORY CAPACITY OF HUMAN MESENCHYMAL STEM CELLS

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The remarkable potencial of human mesenchymal stem cells (hMSCs) for clinical applications has been demostrated in the last years by many studies. The therapeutic activity of hMSCs is carried out thought their strong reparative potential and their ability to display immunomodulatory capacities. We have previously reported that the downregulation of miR-335 is required for the adquisition of the reparative properties (migration, proliferation and differentiation), sugesting that this microRNA is a key regulator of the hMSCs therapeutic activity. Besides their regenerative potential, hMSCs display the capacity of inhibiting the immune response, but very little is known about the molecular mechanisms that are involve in this regulation. The immune supressive action of hMSCs is not constitutive, has to be activated by proinflammatory signals such as the secretion of the proinflammatory cytokine IFN gamma, alone or together with TNF alpha, and other several mediators which induce phenotypic changes of hMSCs. Based in our previous findings, we have demostrated that the activation of hMSCs by IFN gamma or TNF alpha produce the downregulation of miR-335, which is also neccesary for the activation of the immunomodulatory capacity of hMSCs. Moreover, overexpression of miR-335 hMSCs inhibits significantly their immunomodulatory capacity by decreasing their antiproliferative effect over PBMCs in vitro, and the hMSC-mediated downregulation of the inflamatory response in an endotoxemia mouse model *in vivo*. In conclusion, we find that miR-335 functions as a negative regulator of the immunomodulatory properties of hMSCs, suggesting that therapeutic approaches targeting miR-335 could be promissing for the treatment of human pathologies in which inflammation is a major component.

Poster Board Number: F-2319

ROLE OF ALR1/ERV1 IN HUMAN PLURIPOTENT STEM CELL SURVIVAL

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ALR1/Erv1 is a flavin adenine dinucleotide (FAD)-dependent sulfhydryl oxidase that localizes to the intermembrane space in yeast and mammalian mitochondria. In yeast, deletion of ERV1 is lethal, with mutants displaying aberrant mitochondrial morphology, defective protein translocation, and impaired respiration and cell growth. Most intriguingly, ALR1 is one of only a few genes enriched in human embryonic, neuronal, and hematopoietic stem cells. Recently, MB6, a specific inhibitor of ALR1 oxidase activity, was identified in a small molecule screen for activity against mitochondrial function. MB6 blocks protein import of ALR1 pathway substrates into mitochondria and impairs ALR1 substrate oxidation *in vitro*. Here, we show that MB6 selectively induces cell death in human pluripotent stem cells (hPSCs) by cytochrome c release and caspase activation, whereas hPSCs induced to differentiate with retinoic acid were not harmed. Furthermore, MB6 showed no effect on the neural lineage-specific differentiation potential of human iPS cells. Taken together, the data show a critical role for ALR1 mitochondrial redox protein function in maintaining hPSC survival and growth by regulating mitochondrial-mediated apoptosis. Failure of this cell-intrinsic mechanism could lead to the loss of self-renewal or to apoptosis of hPSCs.

Poster Board Number: F-2320

THE DISTRIBUTION OF STEM CELLS IN THE HUMAN AMNION MEMBRANE

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An amnion is embryologically very close to the inner cell mass which is the origin of an embryonic stem cell. Morphologically, an amnion consists of single layer epithelial tissue and connective tissues including mesenchymal cells. The amnion and the other fetal membrane, the chorion, are extend from the edge of the placenta. The amnion is contiguous with umbilical cord and the fetal skin. We reported that a stem cell peculiar to epithelial tissue and connective tissue existed in the amnion membrane and umbilical cord. In this research, the distribution of the stem cells in a human amnion membrane was analyzed. [Materials and Methods] The study was approved by the Ethics Committee, Toyama University. Three places, the umbilical cord circumference, a placenta adhesion part, and a placenta isolation part, were separated, and the freeze them. The whole mount specimen, the paraffin embedded specimen, and the sample for electron microscopes were created. The extracted material was examined histologically and immunohistochemically. As stem cell markers, it was used alkaline phosphatase activity, Oct3/4, OctA, Klf4, Nanog, c-Myc, BCRP, and Sox2. As differentiation markers CK5 and vimentin were used. [Results and Discussion] In the circumference of an umbilical cord, the cells that show a vimentin strong positive to the whole cell were scattered. In the placenta adhesion part and the peripheral zone, almost cells expressed the vimentin positivity to the base side. In the placenta attachment site, one cell or two to three cells expressing Oct3/4 positive were existed, and these cells showed strong ALP activity, too. It was suggested that the amnion stem cells were not uniformly distributed over the whole amnion. And this finding was useful to investigate the stem cell research and clinical usage for their isolation and purification.

Poster Board Number: F-2321

BROWN ADIPOCYTE DIFFERENTIATION OF HUMAN PLURIPOTENT STEM CELLS WITHOUT GENETIC MANIPULATION

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Brown adipose tissue (BAT) is attracting substantial interest due to its possible anti-obesity function. However, direct evidence for its involvement in metabolic improvement has not yet been presented. Moreover, the basic research on human BAT development and the application study on drug discovery remain less progressed

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due to limited provision of human materials suitable for in vitro experiments. Recently, a trial to program human induced pluripotent stem cells (iPSCs) into brown adipocyte (BA) by introducing three transcription factors were reported; however, that system is unsuitable for investigating the natural developmental pathway of BAT. Moreover, functional maturation of the programmed products was not evaluated in that study. To provide a natural way to produce functional human BA, we tried a directed differentiation of human pluripotent stem cells into functional BA without genetic manipulation. Using a specific cocktail, we successfully established a high-efficiency (> 90 %) method to produce highly functional brown adipocyte (BA) from human pluripotent stem cells, including human ESCs and human iPSCs, under a feeder- and serum-free condition (Currently, Japanese patent pending). This is the first success in generating functional BA from pluripotent stem cells without genetic manipulation. The differentiated cells exclusively contained multilocular lipid droplets as confirmed by Oil red O staining. Quantitative RT-PCR studies demonstrated the induction of BAT-specific genes of ucp1 and prdm16, which were undetectable in human mesenchymal stem cell-derived white adipocyte (hMSCdWA). Expression of a series of BAT-selective and BAT/WAT-common genes, but not WAT-selective genes, was also determined by RT-PCR. Immunostaining studies confirmed the expression of UCP1 proteins in mitochondria. Electron micrographs confirmed the presence of multilocular lipid droplets and abundant mitochondria rich in transverse cristae. Functional maturation of hESC/hiPSC-derived BA (hPSCdBA) was also demonstrated in vitro and *in vivo* by subcutaneous transplantation of hPSCdBA into mice. Treatment with an adrenalin β receptor agonist, isoproterenol, augmented the expression of ucp1, a major contributor to thermogenesis. Isoproterenol-responsive thermogenic activation was further confirmed *in vivo*. Respiratory activation was also estimated: we determined significant increments in oxygen consumption rates (OCR) in hPSCdBA in response to an adrenaline β3 receptor-selective agonist, CL316,243, whereas no significant changes in OCR in immature hPSCs and hMSCdWA were detected. The effects of transplantation of hPSCdBA on lipid and glucose metabolisms were also evaluated. hPSCdBA-transplanted mice showed reduced fasting blood triglyceride levels. Olive oil tolerance tests further confirmed that hPSCdBA transplantation augmented resistance to oral lipid loading. In addition, fasting blood glucose levels were significantly lowered in hPSCdBA-transplanted mice compared to saline-injected mice and to hMSCdWA-transplanted mice. These results indicate that hPSCdBA improved both lipid and glucose metabolisms. All these finding together guarantee the production of highly functional and metabolically beneficial BA from hPSC. Our study will make a significant contribution to an advanced understanding of BAT and therapeutic development for obesity.

Poster Board Number: F-2322

NEW METHOD TO DIFFERENTIATE HUMAN PERIPHERAL BLOOD MONOCYTES INTO INSULIN PRODUCING CELLS:HUMAN HEMATOSPHERER CULTURE

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Diabetes mellitus (DM) has been widespread all around the world and more people suffered from DM than ever before. Among two types of DM, type 1 diabetes mellitus (T1DM) is an organ-specific autoimmune disease in which insulin-secreting β-cells are irreversibly destroyed. Up to date, a lot of T1DM patients have been treated with several medications or taken transplantation of pancreatic islets. Although there have been recent advances in T1DM therapy, much still remains to be done. For developing the novel T1DM therapy, insulin producing cells (IPCs) have attracted academic attention for recent years. Several previous studies represented that insulin producing cells (IPCs) could be differentiated from various kinds of stem cells including embryonic stem cells (ESCs) or mesenchymal stem cells (MSCs). However, ESCs and MSCs as the source of IPCs have serious hurdles to be applied clinically such as immunological rejection or limited obtainable amount on using ESCs or MSCs respectively. At this point, human blood born hematosphere (BBHS) from peripheral blood mononuclear cells (PBMNCs) has the distinguishing advantages compared to other stem cell sources: "easily-obtainable autologous cell source". We had previously developed the novel protocol to make BBHS through three-dimensional culture of PBMNCs. And also we demonstrated that BBHS composed of myeloid cells created a stem cell niche for hematopoietic stem cells (HSCs) expansion. Therefore, in this study, we evaluated the potential of BBHS as an autologous source for human insulin producing cells by determining whether insulin positive cells are located or induced in this BBHS. We showed that most of BBHS consist of not only nestin-positive cells which are known as well to have a potential to be differentiated into pancreatic β cell but also small population of insulin positive cells. From this rationale, we investigated research concerning the new protocol for expansion of IPCs derived from BBHS. Under the four-stage protocol for IPCs differentiation, islet-like clusters were formed and most of them expressed insulin. We found that expression of pancreatic specific genes was turned on, such as transcription factors (Pdx-1, Ngn3 and Nkx6.1), genes related to endocrine function (Glut-2 and PC2) or β cell function (Kir6.2, SUR1). Surprisingly, we confirmed not only the existence of IPCs-BBHS through DTZ assay but also the capacity of IPCs to secrete insulin in response to glucose stimulation via GSIS assay. Taken together, our novel protocol successfully induced islet-like human insulin producing cells out of BBHS. This strategy of *ex vivo* expansion of IPCs using BBHS would provide a promising therapeutic method for diabetes.

Poster Board Number: F-2323

THE CLINICAL APPLICATION OF THE NEW DRIED HUMAN AMNIOTIC MEMBRANE (HYPER-DRY AMNION)

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[Aim] Clinical application of human amniotic membrane is limited, especially it is discarded after freezing at three months in Japan. To overcome the limitation, we developed drid human amniotic membrane (Hyper-Dry amnion :HD amnion) and tried clinical application. We evaluated the utility by comparing the treatment using the HD amnion with the current treatment in neurosurgery, otolaryngology, a dental oral surgery, ophthalmology. [Methods] 1) Neurosurgery:We applied it for repair of dura mater deficiency that occurred with surgery. We evaluated it with the treatment using the artificial dura mater. 2) Otorhinolaryngology:We applied it for coating the bone surface that was deficiency of periosteum at the tympanoplasty. We evaluated it with the treatment using the au-

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tologous fascia temporalis. 3) Dental oral surgery:We applied it for the coating to an oral mucosa resection wound and evaluated the effect of treatment. 4) Ophthalmology:We applied it for the coating to an optical surface wound and evaluated the effect of treatment. [Result] 1)Neurosurgery:A good prognosis without a leak of the cerebrospinal fluid was observed. 2)Otorhinolaryngology:The adhesion to a bony wall was high, and there were few infection signs. Also, the tendency that epithelization performed completion (healing) of earlier was observed. 3)Dental oral surgery:A smooth wound surface was formed, and the haphalgesia was almost mild, and it was excellent wound protection. 4)Ophthalmology:The patch therapy with the vital glue of the HD amnion was simple and easy, and was effective for glaucomatous postoperative aqueous humor leakage. [Discussion] Taken together, the method of treatments using the HD amnion, were easy operations, solved the problem that artificial materials remains in the body, and were dominant as compared with current treatment, suggesting that HD amnion is useful for the regenerative medicine.

Poster Board Number: F-2324

IMAGE-BASED CELLULAR ASSAYS FOR GSK3B INHIBITORS AND PRO-NEUROGENIC COMPOUNDS USING IPSC-DERIVED HUMAN NEURONS

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Besides its centrality to many aspects of stem cell biology, the Wnt/GSK3/β-catenin signaling is emerging as a central player in pathways implicated in the pathophysiology of mental illness. Taking the advantages offered by stem cells, such as expandability and specified cell types, we developed several cell-based assays targeting the Wnt/GSK3 pathway using induced pluripotent stem cell (iPSC)-derived human neural progenitors and neurons. We present here an image-based cellular assay using human neurons to measure cellular activity of GSK3 inhibitors. We show that phosphorylation of CRMP2, a GSK3β specific substrate and a protein involved in neuronal differentiation, neuronal polarity, and the regulation of ion channels, is decreased upon treatment with GSK3β inhibitors in iPSC-derived human neurons by Western blotting and immunocytochemistry. The robustness and applicability to compound profiling was demonstrated by screening a library of small molecules targeting Wnt/GSK3 signaling. This assay is GSK3β specific and serves as secondary functional assay in a high-throughput format for GSK3β inhibitors. In addition, we have developed a panel of assays enabling measurement of neurogenesis thereby enabling the characterization and discovery of novel pro-neurogenic compounds. These and other studies hold potential to provide new insights into the molecular mechanisms of neuroplasticity and novel therapeutics for the treatment of neuropsychiatric disorders. Funding provided by NIH/NIMH: R03MH087442, R33MH087896, R01MN091115, and the Stanley Medical Research Institute.

Poster Board Number: F-2325

THE INVOLVEMENT OF P53 IN PROPER FUNCTION OF MOUSE EMBRYONIC STEM CELLS AND INDUCED PLURIPOTENT STEM CELLS

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p53 deficiency enhances the efficiency of mouse somatic cell reprogramming to a pluripotent state. As p53 is usually mutated in human tumors and many mutated forms of p53 gain novel activities, we studied the influence of mutant p53 (mut-p53) on mouse somatic cell reprogramming. Our data indicate a novel gain of function (GOF) property for mut-p53, which markedly enhanced the efficiency of the reprogramming process compared with p53 deficiency. This novel GOF of mut-p53 is not attributed to its effect on proliferation, as both p53 KO and mut-p53 cells displayed similar proliferation rates. Tumors derived from mut-p53 reprogrammed clones were malignant, exhibiting invasive growth, compared to the benign tumors originating from either wild type (WT) or p53 knocked-down reprogrammed cells. In addition, we found that the presence of WT p53 is a prerequisite for the proper selection of cells containing normal karyotype, demonstrating the importance of p53 in preventing abnormal cells from undergoing reprogramming. Furthermore, we demonstrate an oncogenic activity of Klf4, as its overexpression in either p53 KO or mut-p53 cells induced aggressive tumors. Overall, despite the potential of p53 inhibition to enhance reprogramming efficiency, reprogramming of p53-compromised cells was shown to result in the generation of defective iPSCs with chromosomal aberrations and genome instability. These data highlight the importance of p53 as the guardian of all cells, including pluripotent cells. Despite this key role of p53 in pluripotent cells, p53 defective mice are born. This suggests of a mechanism to assure genomic fidelity in spite of the genomic instability expected by a defective or absent p53. This led us to study the possible role of p53 in mouse embryonic stem cells (ESCs). To that end we established and characterized several new ESC lines with various p53 status. Our preliminary results suggest that p53 plays a role in ESCs only under stress conditions. Furthermore, the data we obtained provide a novel insight into the mechanisms underlying the maintenance of genomic stability in embryonic stem cells and in the embryo, in the absence of a WT p53.

Poster Board Number: F-2326

IDENTIFICATION OF EARLY STAGE RENAL PROGENITORS IN E9.5 EMBRYOS BY USING OSR1-GFP KNOCK IN MICE

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Though many groups are trying to induce renal progenitor cells from ES or iPS cells, it remains unclear to which *in vivo* stage those cells correspond. In this study, we propose the stepwise induction model by analyzing the *in vivo* developmental process of renal progenitor cells. Osr1 is one of the earliest markers of the intermediate mesoderm and most of the cell types within the kidney arise from the Osr1+ population. Thus we have generated Osr1-GFP knock-in mice. By sorting Osr1-GFP+ cells from embryos and applying them to the colony- forming assay that promotes renal progenitor differentiation, we have confirmed the existence of renal progenitors in embryos at the early developmental stages. Consecutive application of Osr1-GFP+ kidney precursors to colony forming assay on Wnt4-expressing feeders, from E8.5 intermediate mesoderm to the E11.5 metanephric mesenchyme, revealed that the Osr1+ cells after E9.5 do contain colony-forming cells. Osr1-GFP+ cells at E8.5, however, formed no colonies. Further analyses of expressed genes of colonies derived from E9.5 implied that these colonies are progenitors of mesonephroi and distinct from metanephric progenitors at E11.5. Our data suggests a transition from the primitive to the definitive state between E8.5 and 9.5. These data imply the existence of cues which promote maturation of primitive progenitors to the functional progenitor cells between these developmental stages.

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Poster Board Number: F-2327

IDENTIFICATIONS OF MOUSE MESENTERIC PREADIPOCYTES AND SUBCUTANEOUS PREADIPOCYTES

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Since the concept of the metabolic syndrome was proposed in 1987, many reports have shown that visceral fat accumulation and dysfunction are major causes of metabolic syndrome, and there are distinct metabolic and biologic properties in visceral and subcutaneous adipose tissues. However, most of these papers were based on clinical, epidemiological, or physiological analysis, and molecular mechanism underlying these different adipocytes have been unclear. In a conventional method, mature adipocytes can be isolated from visceral or subcutaneous fat pad, and many culture techniques have been proposed until now, however, mature adipocytes are fragile, short-lived and contaminated with other cell types including immune cells or stromal cells, moreover, these techniques are not general and poorly-reproducible. Therefore, we aimed to identify preadipocytes from mouse mesenteric and subcutaneous adipose tissues, which can proliferate and differentiate into adipocytes in vitro. At first, we examined the expression patterns of surface markers in stromal-vascular fraction (SVF) of mouse mesenteric and subcutaneous fat tissue, which is a heterogeneous mixture of cells including fibroblasts, vascular endothelial cells, and immune cells. Using FACS with more than 70 kinds of antibodies whose targets are known to stem cell markers, we found that CD29 and CD34 double positive fibroblasts of SVF expressed more than 20 stem cell markers, including CD90, BP-1, etc.. To elucidate whether these molecules have a role of the surface markers of preadipocytes, we sorted the SVF cells into the each molecule-positive or -negative fraction, cultured sorted cells and induced differentiation to adipocytes. Finally, we identified the surface markers of mouse mesenteric preadipocytes (M-pre) and subcutaneous preadipocytes (S-pre), and found the difference of the marker between M-pre and S-pre. Next, to investigate whether two types of mature adipocytes from M-pre and S-pre maintain each characteristic phenotype in vitro as reported in adipose tissues *in vivo*, we assessed lipolytic activity of adipocytes from M-pre and S-pre. The adipocytes from M-pre had higher lipolytic activity than those from S-pre, and these data indicate that in vitro mesenteric and subcutaneous adipocytes keep the different characters as well as *in vivo* respective adipocytes. In conclusion, we identified M-pre and S-pre in mesenteric and subcutaneous fat tissues, respectively. And, using these cells, we established a new versatile experimental system to compare mesenteric adipocytes with subcutaneous adipocytes in vitro.

Poster Board Number: F-2328

A PAINFUL EXPERIENCE IN STROKE: CHARACTERIZATION OF BEHAVIORAL AND CELLULAR STRESS CORRELATES IN ADULT RATS EXPOSED TO EXPERIMENTAL STROKE

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Objective: Stress has been implicated as a major exacerbating factor for stroke pathological manifestations, including immune response. The present study was designed to investigate the interaction between stroke and stress, with the latter characterized behaviorally by a novel pain perception scale, and cellularly by DNA damage in the thymus, an important organ for immune-defense system. Methods: Sprague-Dawley rats were randomly assigned to middle cerebral artery occlusion (MCAo) group and intact control group. Routine behavioral and histological assays were performed to determine stroke symptoms. For characterization of stress, the subjects were videotaped for 15 min at 1 day before and 0, 1, and 3 days after MCAo. After video recording at day 3, all animals were sacrificed for collecting their blood and thymus. Subjects' pain perception, which is considered as one of post-stroke stressors, was analyzed from the video in accordance with Rodent Grimace Scale. Concentration of plasma corticosterone was analyzed with an ELISA kit. DNA damage in the thymus was analyzed by immunostaining with gamma-H2AX antibody, which is known as DNA double strand break marker. Results: Stroke animals displayed the characteristic motor asymmetry and cerebral infarction, whereas intact control subjects exhibited normal behaviors and non-detectable pathological damage to the brain. Pain scores before MCAo revealed no significant differences across groups. Following MCAo, stroke subjects showed significantly higher pain scores than those in intact subjects ($p < 0.05$). Concentration of plasma corticosterone was also higher in stroke subjects than those in intact subjects ($p < 0.05$). Gamma-H2AX immunoreactive cells were localized at the boundary between medulla and cortex of the thymus, and were highly expressed in the stroke group than the control group. Conclusions: This is the first report extending the utility of the rodent grimace scale in a neurological condition. Here we show that the typical stroke neurobehavioral symptoms produced by MCAo were accompanied by a pain response. Moreover, we demonstrate that this stroke-induced behavioral and physiological stress response coincided with an upregulation of a cellular stress marker involving DNA damage in the thymus. Stress is also a well established inhibitor of cell proliferation in the hippocampus. Stem cell treatments after experimental stroke, at least in the acute setting, may benefit from a careful consideration of treating stressors.

Poster Board Number: F-2329

NEURAL DIFFERENTIATION RATE OF MOUSE EMBRYONIC STEM CELLS DECREASED BY PEROXISOMAL PROTEIN EXPRESSION REDUCTION

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Peroxisomal protein (PEP) was initially cloned by Ferrer-Martinez in 2002 and later by our group in 2009.We showed that its expression increases, upon retinoic acid treatment in neural differentiation of mouse embryonic stem cells. To assess the importance of the PEP in neural differentiation, a doxycycline inducible eukaryotic expression vector was implemented for efficient production of short hairpin *PEP* RNA in stably transformed mouse embryonic stem cell (mESC) line. The *PEP* knockdown was carried out in both

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during neural progenitor (NPs) formation and also during neural differentiation . Interestingly, the decrease in *PEP* expression during NPs formation significantly reduced expression of NPs and mature neuronal markers. On the other hand, *PEP* knockdown during the aforementioned stages significantly affected both neuronal and astrocytes maturation, concluding that *PEP* expression is required for the appropriate neural differentiation of mESCs. These data have confirmed recently published reports that describe the importance of peroxisomes and their contents in the generation and development of the nervous system.

Poster Board Number: F-2330

ADULT DENTAL PULP DERIVED STEM CELLS: AN ALTERNATIVE SOURCE FOR *IN VITRO* GENERATION OF NEURONS

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Human Mesenchymal Stem Cells (hMSCs) are multipotent stem cells and can be isolated from different sources like dental pulp, umbilical cord, bone marrow (BM), adipose tissue etc. However, BM-MSC is considered as one of the best candidates for regeneration of cells because of their properties like easy isolation, robust expansion, immunological naivety and absence of ethical issues. However, isolation of BM is a painful procedure; therefore it calls for an easy accessible source of Stem Cells (SC). Keeping this in mind, the study was designed to explore the differentiation potential of Dental Pulp Stem Cells (DPSC) specifically into neurons and its comparison with BM-MSC. Study was initiated after the approval from Institute Ethics Committee (IEC) & Stem Cell Ethics Committee. Cryopreserved BM-MSCs were used in this study. Discarded normal human impacted first premolars were collected from patients (12-19 years of age (N=5)) with informed consent of patients undergoing routine extractions at the Centre for Dental Education and Research, AIIMS. Tooth surfaces were cleaned and cracked open using a bone cutter to reveal the pulp chamber. Pulp was than washed with PBS and placed in culture dishes for explants culture, after gentle teasing, in LG-DMEM supplemented with 10% FBS at 37ºC/5%CO2. Initiation of cell growth was observed within 10-15 day of culture. Cells were expanded and cryopreseved at every passage. All the experiments were performed using 3rd passage cells after their characterisation. Morphologically, they were adherent, spindle shaped and grew in monolayes. They were checked for the presence of vimentin and Fibroblast Specific Protein (FSP) (Immuno fluorescence (IF) assay); Surface markers -CD105, CD90, CD29, CD73, HLA I & II and CD 34/45 by Flowcytometry. DPSC & BM-MSC were induced to differentiate into osteo- , adipo- and chondrocytes. Their proliferative potential was assessed by MTT assay. After characterization, the cells were differentiated *in vitro* into neurons using an induction medium consisting of Neurobasal media, B27 supplement, Basic fibroblast growth factor (bFGF), Epidermal growth factor (EGF), L-Glutamine and Antibiotic for 12 days. Morphological changes were observed and differentiated cells were characterized by RT-PCR and IF studies for neural specific markers - Nestin, Neurofilament (NF), Beta III tubulin (TUJ I), Microtubule-associated protein 2 (MAP2). These cells were positive for vimentin, CD105, CD90, CD29, CD73, HLA I and negative for FSP, HLAII and CD34/45. They were easily induced into osteocytes and chondrocytes. All the above features strongly suggest that DPSC were equivalent to MSC with higher proliferative rate, However they fail to differentiate into adipocytes. Differentiation studies revealed the expression of Nestin, NF, TUJ I, MAP2 at basal level which increased after treatment with induction media (IF and RT-PCR). Semi-quantative RT-PCR analysis revealed higher neuronal gene expression level in DPSC than BM-MSC; with highest expression of MAP-2 followed by TUJ-1. These Initial studies reveal that DPSC are equivalent to BMSC & can be a better candidate for regenerative medicine due to their easy accessibility, proliferation rate and differentiation potential. Further analysis by q-PCR and functional studied of differentiated neurons is ongoing and will contribute to the existing information.

Poster Board Number: F-2331

INVESTIGATION OF RELATIONSHIP BETWEEN THE SCAFFOLD PORE SIZE AND THE CHONDROGENIC DIFFERENTIATION OF RABBIT ADIPOSE-DERIVED STEM CELLS

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Scaffolds play an important role in maintaining the phenotype of chondrocyte in cartilage tissue engineering. Polycaprolactone (PCL) is one of the most widely used biodegradable polyesters for medical applications owing to its biocompatibility, biodegradability and flexibility. Scaffolds fabricated using PCL are more resistant to hydrolysis due to slower degradation, and maintain long-term structural integrity during the in vitro culture period. The purpose of this study is to investigate the relationship between the pore size and the chondrogenic differentiation of rabbit adipose-derived stem cells (ASCs). For this, we fabricated the pore size gradient PCL cylindrical scaffold, seeded rabbit ASC into the scaffold, and analyzed the chondrogenic gene expression. We also investigated that the difference of scaffold pore size can improve osteochondral repair in *in vivo* rabbit chondral defect model. In this study, our in vitro results indicate that the ASCs may prefer larger pores for chondrogenic differentiation than the smaller pores, even though *in vivo* results were not effective. Therefore we suggest that pore size gradient scaffolds fabricated by a modified centrifugation method should contribute to the determination of optimum pore size ranges for a variety of stem cell differentiation to specific cell types.

Poster Board Number: F-2332

MICRORNAS REGULATE THE CELL CYCLE IN HESCS THROUGH RBL1 AND RBL2 USING TWO DIFFERENT REPRESSIVE MECHANISMS

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Human embryonic stem cells (hESCs) are enriched for several families of microRNAs (miRNAs), but their specific targets and functions remain largely unknown. The seed sequence similarity among the highly expressed hsa-miR-302, hsa-miR-372, and hsa-miR-17 families suggests that repression of their targets may be critical for maintaining the hallmark characteristics of hESCs. Here, we show that potent targeting of the Retinoblastoma-like (RBL) proteins by all three miRNA families is necessary for the unusually rapid G1 to S transition unique to the hESC cell cycle. Unexpectedly, the mecha-

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nism of miRNA-mediated post-transcriptional regulation was different for the two RBL proteins. RBL2 was repressed via the expected mechanism of mRNA destabilization, but RBL1 was regulated by translational repression, a novel example of this mechanism in mammalian cells. This work shows that highly expressed miRNAs regulate the cell cycle in hESCs by targeting two members of the same gene family through different repression mechanisms.

Poster Board Number: F-2333

EXPRESSION OF CELL-SURFACE ANTIGENS IN MARMOSET EARLY EMBRYOS

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Common marmoset is an only non-human primate that can produce genetically modified animals. Although several marmoset Embryonic Stem (ES) cells have been established, their pluripotency have not been confirmed because molecular mechanisms for maintaining pluripotency in non-human primate embryos and ES cells are not well known. Therefore target gene knockout non-human primate including marmoset has not been produced. If the target gene knockout non-human primate would be produced it would contribute to wide area of life science research. The expressions of cell-surface antigens in ES cells, and early embryos in mouse and human have been reported, however there is no information about the non-human primate including marmoset at the early embryos. Especially, stage-specific-embryonic-antigen-1 (SSEA-1) expression is observed in mouse ES cells but not in that of primates and recent studies suggest that SSEA-1 expression is related to pluripotency of mouse ES cells. Therefore cell-surface antigens would be used as an invaluable tool for the analysis of cell type and the cell differentiation. In this study, we have evaluated the expression of cell-surface antigens including SSEA-1, SSEA-4, TRA-1-60 and TRA-1-81 on the marmoset pre-implantation embryos. Marmoset embryos at 8-cell, 16-cell, morula, compacted morula and blastocyst stages were collected for the immunostaining. The SSEA-1 expression was observed at 8-cell, 16-cell stage, and morula but not observed from compacted morula through expanded blastocyst stage. On the other hand, SSEA-4 expression was observed in ICM of the blastocysts. TRA-1-60 expression was shown at the morula and the blastocyst stages, but not observed expanded blastocyst stage. TRA-1-81 expression was found at the expanded blastocyst stage. In the marmoset early embryos, the expressions of cell-surface antigens are associated with dynamic molecular expression profile changes in the developing embryos. This founding is a first step to understand non-human developmental biology.

Poster Withdrawn

Poster Board Number: F-2335

TRANSGENIC PIGS WITH PANCREAS AND LIVER SPECIFIC EXPRESSION OF FLUORESCENT PROTEINS

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Genetically modified pigs expressing fluorescent proteins provide invaluable research tools for bio-medical researches including cell transplantation, tissue engineering and organ regeneration. The aim of this study was to develop transgenic pigs characterized by pancreas and liver specific fluorescence expression. A transgene construct expressing Venus (green fluorescence) under control of mouse Pdx1 (pancreatic duodenal homeobox-1) promoter and a BAC derived construct consisting of porcine albumin promoter and humanized Kusabira-Orange (huKO, red fluorescence) was introduced into porcine *in vitro* matured oocytes using intracytoplasmic sperm injection (ICSI)-mediated gene transfer method. Transfer of 370 Pdx1-Venus embryos into 4 recipients gave rise to 22 fetuses and piglets of which 9 exhibited pancreas specific Venus expression. After transfer of 523 Alb-huKO embryos into 4 recipients, 19 piglets were obtained. Of these, a female piglet was confirmed to be transgenic and showed liver specific huKO expression. This founder transgenic female produced a total of 5 transgenic and 12 non-transgenic offspring in 2 litters after mating with a wild-type boar. Liver specific huKO expression was inherited to these offspring. Furthermore mating of a female Pdx1-Venus with an Alb-huKO boar yielded 10 transgenic pigs including 4 carrying both transgenes. These double-transgenic pigs exhibited both the pancreas specific Venus and liver specific huKO expressions in single individuals. These data demonstrate that transgene introduction by the ICSI-mediated gene transfer into *in vitro* matured oocytes is

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a feasible method to generate pigs expressing fluorescent proteins in a tissue specific manner. Transgenic pigs with pancreas-specific-green plus liver-specific-red fluorescence would provide a useful large animal model for various researches such as stem and progenitor cell therapy and tissue/organ generation. This study was supported by JST, ERATO, Nakauchi Stem Cell and Organ Regeneration Project.

Poster Board Number: F-2336

EXPLORING THE LANGUAGE OF CELL IDENTITY THROUGH BIOINFORMATIC RECONSTRUCTION OF TRANSCRIPTIONAL REGULATORY CIRCUITS IN THE MAMMALIAN NERVOUS AND IMMUNE SYSTEMS

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Much of the promise of regenerative medicine is based on the idea of controlling or redirecting cell fate. Although we have achieved remarkable success in reprogramming cells through expression of transcription factor combinations, we lack a computational and theoretical framework for decoding or systematically manipulating the transcription factor regulatory circuits that control cell identity across adult cell types. Here, I develop a bioinformatic framework for deriving predictive models of transcriptional regulatory circuits that control cellular identity in the adult mouse nervous and immune systems. By combining techniques from information theory with comparative methods from evolutionary biology, I derive reduced models of transcription factor circuits across a large number of cell types. These models provide maps for studying the architecture of cellular diversity in two important physiological systems. In this poster, I discuss the use of probabilistic methods for construction of cell identity models from high-throughput data . Second, I relate qualitative features of the cell fate model's structure to potential design principles of cellular identity and describe the transcription factor regulatory circuits in the cortex, amygdala, cerebellum, and hippocampus as well as the B-cell, T-cell and Natural Killer cells. Third, the nervous system and immune system have undergone rapid diversification in vertebrates, and I connect the architecture of the cell identity model in the mouse to gene expression patterns in human and zebrafish to hypothesize potential mechanisms of cellular diversification through gene family expansion. Finally, I describe an algorithm for using the probabilistic model as a hypothesis generation tool for forward cell programming experiments. Reprogramming and transdifferentiation have become important tools for generating cell types and for testing our knowledge of transcriptional regulatory circuits that control cellular identity. Comprehensive maps of cell fate circuits provide a framework for predicting and refining transcription factor combinations for programming specific types of neurons and immune cells.

Poster Board Number: F-2337

EFFECTS OF THE INTRAVENOUS BONE MARROW MONONUCLEAR CELLS THERAPY IN STROKE MODEL

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Stroke is the second leading cause of death in the world and the largest cause of disability in adults. Cell therapy has been shown to have beneficial effects in animal models of stroke. In this respect, we have previously shown functional improvement after bone marrow mononuclear cells (BMMCs) transplantation, either by intra-arterial or intravenous routes. Moreover, we observed that the number of cells that migrate to the ischemic hemisphere is very low, in accordance to other works. This small number of cells found in the cerebral parenchyma suggest that peripheral mechanisms may play a systemic role in cell therapy, i.e., the cells do not necessarily need to reach the central nervous system in order to trigger their therapeutic effects. In this work, we used the model of stroke by thermo-coagulation of blood vessels in sensorimotor cortex of adult rats to investigate the possible systemic therapeutic effects of the BMMCs. BMMC-treated animals had an increase of the Ki-67+ cells in the subventricular zone (SVZ) in the hemisphere ipsilateral to the lesion 7 days post-ischemia (PID; n=6, $p<0.05$) compared with saline group. In this same period, the activated microglia was evaluated through the analysis of CD68 antigen in the periphery of the lesion in treated and untreated animals. We observed that the number of activated microglia was reduced in the animals treated with BMMCs when compared with control group (n=4; $p<0.05$). To evaluate the systemic effect of the BMMCs therapy we performed the removal of the spleens. The splenectomy was followed 2 weeks later by ischemia and after 1 day the animals received injections of the BMMCs or PBS. The functional behavior was assessed using rotarod and cylinder tests. The rats that underwent brain injury with or without splenectomy presented deficits in both tests. The splenectomized rats subjected to stroke followed by BMMCs treatment showed a marked decrease in the functional performance when compared with nonsplenectomized rats (n=6; $p< 0.001$). This data suggest a important role of the spleen for the intravenous cell therapy.

Poster Board Number: F-2339

HETEROGENEITY OF NEURAL CREST STEM CELLS REVEALED BY THEIR MULTILINEAGE DIFFERENTIATION POTENTIALS AND GENE EXPRESSION PROFILES

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Recent reports have described that neural crest-derived stem cells (NCSCs) are self-renewing and multipotent. We previously reported the existence of multipotent NCSCs in the dorsal root ganglia (DRG), wisker pad (WP), and bone marrow (BM) of adult rodents. However, the characteristics of NCSCs derived from these tissues have not been elucidated yet. Here we found difference of characteristics in the NCSCs among neonatal DRG, WP and BM with the transgenic mice harboring neural crest-specific reporter, P0-Cre/Floxed-EGFP. EGFP-positive spheres derived from three tissues differentiated into neural crest lineages which include neurons, glial cells and myofibroblasts. While EGFP-positive sphere derived from WP and BM differentiated into adipocytes, EGFP-positive sphere derived from DRG did not differentiate into adipocytes. It is reported that differentiation of glial cells is higher in EGFP-positive sphere derived from DRG than those of WP and BM in adult rodents. Therefore, we investigated the extent of CpG methylation in the Gfap promoter, including the STAT3-binding site, and S100β promoter, which are related in glial differentiation. The extent of CpG methylation in the Gfap promoter and S100β promoter was significantly lower in EGFP-positive sphere derived from DRG than those of WP and BM. Therefore, differentiation into glial lineages from NCSCs may be controlled by epigenetic modification. Gene

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expression profiles among EGFP-positive sphere derived from DRG, WP and BM of neonate and adults were analyzed by with the Agilent 2100. Our results revealed that principal component analysis was significant tissue-source-dependent difference. In the future, identification of differentiation of NCSCs derived from various tissues will provide a new potential source for autologous cell therapy after nerve injury or disease.

Embryonic Stem Cell Pluripotency

Poster Board Number: F-3001

COMPARISON OF HPSC CULTURED IN MTESR™1 AND E8 ALBUMIN-FREE MEDIA UTILIZING ENZYME FREE PASSAGING METHODS

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The maintenance and propagation of human pluripotent stem cells (hPSC) in feeder-independent conditions for multiple passages requires the use of complex media formulations, in combination with careful handling techniques, to maintain high quality cultures at each passage. Recent efforts have focused on improving the general utility and reproducibility of hPSC maintenance culture protocols by developing new and more straightforward strategies that increase consistency by the removal of undefined components and reagents from the culture system. A simplified version of the commercially available mTeSR™1 and TeSR™2 hPSC maintenance media formulation, referred to as E8, has recently been reported by the Thomson lab. E8 was developed via the pairwise removal of extraneous additives from the TeSR™ core media formulation, resulting in an albumin-free media with a minimum set of components. We sought to compare E8 with mTeSR™1 using multiple hPSC cell lines (H1 hESC, H9 hESC and a commercially available episomal hiPSC Line) and to optimize protocols for culturing hPSC using E8 in the presence of Matrigel™. To initiate these experiments, hPSCs from mTeSR™1 maintenance cultures were seeded into Matrigel™ coated plates containing either E8 or mTeSR™1 media. Cells were passaged every 5-7 days for up to 8 passages using an enzyme-free passaging method. Briefly, cells were dissociated by the removal of media and the addition of Gentle Cell Release Buffer (STEMCELL Technologies Inc.) for 6 minutes. The buffer was removed and replaced with media prior to detachment of the cells via scraping. Proper care was necessary to minimize clump breakup while cells were transferred to a fresh plate. At each passage, cells were characterized by assessing plating efficiency, colony morphology and cell expansion. We demonstrated that using this protocol, we could routinely achieve high expansion of total cells in E8 with H1 (11 ± 1 fold; mean ± SD n=5 passages), H9 (16 ± 2 fold; n=8 passages) and the episomal hiPSC Line (7 ± 1 fold; n = 5 passages). To enable the success of achieving long term cultures reproducibly, it was critical that key steps in the passaging protocol be tightly regulated. For example, improper handling techniques during passaging resulted in poor performance of cultures marked by low cell attachment and high cell differentiation. To determine the immediate effects of transitioning hPSCs to E8 albumin-free media from mTeSR™1 albumin containing media, we assessed plating efficiencies and expansion rates obtained for the passages immediately following transition. We found that there was little change in expansion rates upon transitioning from mTeSR™1 to E8 media, although the plating efficiency was generally lower than normal (17 ± 7%, n =3) in the passage immediately following the transition; the plating efficiencies approached expected levels (40-100% per passage) 2-3 passages after transfer. Overall, these experiments revealed key similarities between hPSC maintained in E8 and mTeSR™1 media in the presence of Matrigel™, and the transfer of cells from mTeSR™1 to E8 resulted in a temporary drop in plating efficiency as cells adapted. Our findings demonstrated that E8, an albumin-free medium formulation, is a good alternate media for hPSC maintenance, and when combined with optimized protocols is capable of maintaining hPSC with behavior consistent with those maintained in mTeSR™1 and TeSR™2 media.

Poster Board Number: F-3002

TRANSITION OF HUMAN PLURIPOTENT STEM CELLS CULTURED ON MATRIGEL™ TO STEMADHERE™ DEFINED MATRIX FOR HPSC

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The culture matrix is one of the key requirements in any feeder-free based culture system designed to support consistent and reproducible long-term expansion of human pluripotent stem cells (hPSC). To date, hPSC cultures using defined media such as mTeSR™1 or TeSR™2 have typically necessitated the use of crude preparations of extracellular matrices (ECMs) to permit cell attachment. Although Matrigel™ (BD Biosciences) is undefined and derived from a mouse tumor, it is the most commonly used matrix in mTeSR™1 and TeSR™2 hPSC maintenance cultures. The main components of Matrigel™ are collagen and laminin, both of which are known to interact with cells via surface integrin receptors. Although integrin mediated attachment through collagen and laminin is well understood, the routine use of purified collagen and laminin matrices available from various suppliers has been hampered by the batch-to-batch variability and high costs of these ECMs. StemAdhere™ Defined Matrix for hPSC (developed and manufactured by Primorigen Biosciences) is a defined human recombinant protein matrix that supports expansion of human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) in defined, feeder-independent maintenance culture conditions. StemAdhere™ interacts with extracellular E-cadherin that is expressed on the surface of hPSC to promote cell attachment onto the surface. Using an enzyme-free protocol, we demonstrate that this alternative E-cadherin mediated attachment system allowed for long term passaging of hPSC in mTeSR™1 or TeSR™2. These protocols were optimized to allow the convenient transition of hPSCs previously cultured in the presence of Matrigel™ to StemAdhere™. We observed that hPSC cultured in mTeSR™1 or TeSR™2 on StemAdhere™ initially undergo an "adaptation phase" for 2 to 4 passages in a manner similar to that observed when transitioning hPSC previously maintained on feeder cells to feeder-independent conditions. During the adaptation phase, the colony morphology of hPSC cultured in mTeSR™1 or TeSR™2 on either Matrigel™ or StemAdhere™ is comparable, however some subtle morphological differences are observed. After the adaptation phase, hPSC cultured in mTeSR™1 or TeSR™2 in the presence of StemAdhere™ exhibited plating efficiency (60-100% per passage) and expansion rates (8-12 fold expansion per passage) equal to or greater than those seen for hPSC maintained in the same media on Matrigel™ or other similar matrices. Multiple lines of hESCs and hiPSCs were successfully passaged in long term maintenance cultures (≥ 20 passages) containing mTeSR™1 or TeSR™2 media in the presence of the StemAdhere™. hPSCs from these long term cultures maintained high expression (>90%) of the pluripotency markers SSEA3 and OCT4, remained karyotypically normal, and generated all three expected germ lineages in teratoma formation assays. Our results demonstrate that hPSCs maintained on StemAdhere™ in the

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presence of mTeSR™1 or TeSR™2 responded predictably in standard characterization analyses and behaved functionally equivalent to hPSCs maintained on the undefined Matrigel™ matrix. StemAdhere™ is therefore a suitable matrix for routine maintenance of hPSC under defined and animal component free conditions.

Poster Board Number: F-3003

ROBUST EXPANSION IN MONOLAYER REVERSIBLY ELEVATES VIABILITY OF HUMAN EMBRYONIC STEM CELLS

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The need for a robust, invariable, and cost-effective culture of human embryonic stem cells (hESCs) resulted in several large-scale systems. However, the novelty was often hampered by changes in hESC phenotype, genomic instability, or difficult manipulation. Here we report a comprehensive analysis of pluripotency features by hESCs, which have been propagated for over 100 passages in monolayer (ML) culture. The ML system is based on single-cell dissociation and plating of cells in high densities on a matrix-coated surface, so they become fully confluent within 72 hours. This results in homogenization of the cell population and efficient expansion with 1:20 ratio every 3 days. While the in vitro and *in vivo* differentiation capacity remained to be the same, successful development of a teratoma required 5-times lower input of hESCs from the ML system then of their feeder-dependent counterparts. This indicates (a) different portion of teratoma initiating cells or (b) different survival rate early after injection. As the presence of SSEA-5- and Oct-4- positive cells was comparable in both culture systems after differentiation, teratoma development from ML hESCs was not driven by an increased pool of differentiation-resistant cells. Also, we found none of the recently described adaptation-related proteins (Bcl-xL, Bcl-2 and survivin) to be overexpressed in ML hESCs. In two viability assays, ML hESCs showed superior survival and participation to assays than colony-dependent culture. Finally, we found that the reverted feeder-dependent culture displayed normal phenotype of the colony growth, gradually developed back the dependence on Y27632 in viability assays, and lowered teratoma efficiency towards the ground state represented by feeder-dependent hESC colonies at the beginning. In addition to the stable karyotype, the reversibility rather points to an unselective process of adaptation and challenges the hypothesis of clonal expansion in ML system. In addition to that, a simple automation and compatibility with xeno-free conditions make the ML system highly suitable for production of vast numbers hESCs necessary in cell-based therapies.

Poster Board Number: F-3004

MULTIPLE LARGE DELETIONS IN THE MITOCHONDRIAL DNA OF HUMAN EMBRYONIC STEM CELLS

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Mitochondria are one of the key elements of the unique biology of stem cells. Undifferentiated human and mouse embryonic stem cells (ESC) contain few, spherical and immature mitochondria, and their number and maturity increases upon differentiation, concurrent with the switch from glycolysis to oxidative phosphorylation (OXPHOS) for energy production. Furthermore, attenuating mitochondrial function in undifferentiated hESC increases the mRNA levels of the pluripotency genes NANOG, POU5F1 and SOX2, compromises their differentiation potential and increases the number of persisting tumorigenic cells after differentiation. Despite the increasing number of reports on the high instability of the nuclear genome of hESC and the clear role of mitochondria in maintaining the pluripotent state, little attention has been dedicated to the integrity of the mitochondrial genome. In this study, we screened for mtDNA deletions in one to six different passages of eighteen hESC lines, the passage number ranging from passage three to 334. We also studied DNA from hESC differentiated to the osteogenic progenitor lineage, to the lung epithelium lineage and spontaneously differentiated in embryoid bodies and on feeder layers. Single-cell derived hESC lines were created to investigate the variety of deletions within one cell. The mtDNA was fully amplified by PCR using different combinations of 8 primers, generating fragments between 6 and 10 Kb in wild type mtDNA. The PCR products were visualized by agarose gel electrophoresis and the specificity of the PCR was confirmed by Sanger sequencing. All analysed undifferentiated and differentiated hESC samples showed deletions, even in passages as early as three and six. In some lines we could identify over a dozen of deletions, their sizes up to 8,4 Kb. Moreover, numerous deletions coexisted in clonal hESC lines, indicating that probably each individual hESC contains various mutated mtDNA molecules. We established the breakpoints of twenty-two deletions by sequencing, which revealed that most of them (20/22) were flanked by repeats. Seven of these deletions coexisted in one clonal hESC line. Strikingly, the deletions observed in hESC are not restricted to the common region of deletion seen *in vivo* in the human, but appear all over the mitochondrial genome. Although difficult to establish, the mutation load seems to be variable and low. Two non-mutually exclusive hypotheses could explain the origin of these mutations. Since different mutations are already present at very early passages, it is possible that they originate from the embryo that was used for the derivation of the line. Another possibility is that the deletions appear spontaneously in culture due to low expression levels of genes involved in the mtDNA replication and repair. In summary, all analysed hESC lines and their differentiated derivatives carry a plethora of mtDNA mutations. This is the first time this has been described and adds a new layer to the known instability of the stem cell genome.

Poster Board Number: F-3005

THE ROLE OF FIBROBLAST GROWTH FACTOR RECEPTOR1 IN HUMAN ESC ADHESION SIGNALLING AND PROLIFERATION

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Basic fibroblast growth factor (FGF-2) promotes self-renewal, survival, and adhesion of human ESCs. The diverse functions of FGF-2 may be attributed to its interactions with various fibroblast growth factor receptors (FGFRs), which in turn lead to different signalling cascades. Our study explores the role of FGFR1 in the human ESC adhesion, signalling and proliferation. FGFRs are tyrosine kinase receptors involved in a broad range of developmental processes, but their functions or expression often is dysregulated in developmental malignancies and cancers. Our data showed that hESCs express all types of FGFRs (1, 2, 3 and 4), to which FGFR1 is the most abundant. Intriguingly, the phosphorylation induced by FGF-2 treatment in FGFR1 is the least amongst the FGFRs. Our results sug-

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gest that the tepid reaction of FGFR1 to FGF-2 in phosphorylation implicates the action of FGFR1 in non-canonical FGF pathway. We also observed that FGFR1 localises mainly in the cell membranes where the cell-to-cell contacts occur. We also found that FGFR1 co-stains with ZO-1 protein, which is involved in tight junction assembly. Interestingly, in lower density cultures, we observed that FGFR1 and ZO-1 co-localise in nucleus in addition to the location in the cell membrane. Our data suggest that FGFR1 may play a role in cell adhesion and contact inhibition. Herein, we propose a potential novel role of FGFR1 in the microenvironment of human ESC interactions.

Poster Board Number: F-3006

ENGINEERING CELL MATERIAL INTERFACE FOR LONG TERM EXPANSION OF HUMAN PLURIPOTENT STEM CELLS

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Developing cost-effective, and scalable synthetic matrices for long-term expansion of human pluripotent stem cells (hPSCs) is important to realize their applications, ranging from drug screening platforms to regenerative medicine. Here, we report the development of a synthetic matrix containing heparin mimetic moieties to support long-term expansion of hPSCs for at least over 20 passages in a chemically defined Stempro medium. HPSCs expanded on these synthetic hydrogels maintained their characteristic morphology, colony forming ability, karyotypic stability, and differentiation potential. The synthetic matrix-expanded hPSCs exhibited pluripotency markers comparable to those cultured on Matrigel. Employing the hydrogel-based synthetic platform, we also investigated the effect of various physicochemical properties of the matrix (e.g., functional group, hydrophobicity, and matrix rigidity) on adhesion, growth, and self-renewal of hPSCs. Our findings suggest that the synthetic matrices having an optimal interfacial hydrophobicity and matrix rigidity support long-term self-renewal of hPSCs. The observed cellular responses are explained in terms of matrix mediated binding of extracellular matrix proteins and growth factors, which provide a conducive microenvironment for the initial adhesion and growth of hPSCs. Such synthetic matrices comprising of "off-the shelf" components are easy to synthesize and do not require any sophisticated processing thus making them cost-effective and translational. Furthermore, synthetic matrices with defined bulk and interfacial properties will be an ideal tool to understand the molecular mechanisms that control fate and commitment of hPSCs.

Poster Board Number: F-3007

RECOMBINANT E8 FRAGMENTS OF HUMAN LAMININ ISOFORMS SUPPORT THE EFFICIENT ADHESION AND EXPANSION OF DISSOCIATED HUMAN PLURIPOTENT STEM CELLS UNDER DEFINED AND XENO-FREE CONDITION.

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Human pluripotent stem cells (hPSCs), including human embryonic stem cells and induced pluripotent cells, have the potential to provide infinite sources of tissues for regenerative medicine. Although defined and xeno-free media have been developed, culture conditions for reliable propagation of hPSCs still require considerable improvement. Here, we show the long-term undifferentiated culture of dissociated hPSCs using recombinant E8 fragments of laminin isoforms (LM-E8s). The LM-E8s are the functional minimal forms that retain full integrin binding activity, and they are easier to be produced as the recombinant proteins than intact laminins. Small volume of LM-E8s was sufficient to coat the culture vessels, but the LM-E8s promoted greater adhesion of hPSCs than intact laminin isoforms and Matrigel. We successfully maintained four hPSC lines on the LM-E8s in three defined media for 10 passages with single-cell passaging. Furthermore, we could culture the dissociated hPSC on the LM-E8s in defined and xeno-free medium for over 30 passages, sustaining a high level of expression of pluripotent markers, a normal karyotype, and the potential of differentiation into all three germ layers. This culture system using the LM-E8s with single-cell passaging allows the robust expansion of hPSCs for therapeutic applications.

Poster Board Number: F-3008

CROSSTALK BETWEEN TGF-BETA AND BMP SIGNALING IN PLURIPOTENT HUMAN ES CELLS

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Human embryonic stem cells (hESCs) and mouse epiblast stem cells (mEpiSCs) require basic FGF and TGF-beta/Activin signaling to maintain pluripotency. The molecular mechanisms of how TGF-beta/Activin signaling maintains stem cell pluripotency remain unclear. Upon TGF-beta/Activin stimulation, both Smad2 and Smad3 are activated through phosphorylation, and translocate into nuclei to regulate target gene expression. Specific inactivation of the TGF-beta/Activin type I receptors by kinase inhibitors (i.e. SB431542) inhibits Smad2 and Smad3 phosphorylation, as well as non-Smad signaling mechanisms, and does not allow us to understand the contributions of specific pathways in the establishment and maintenance of pluripotency in hESCs or mEpiSCs. TGF-beta/Activin-activated Smads play important roles in mesoendodermal differentiation, and positively regulate one of the pluripotency transcription factors, Nanog, by binding to its proximal promoter. Since Smad2 and Smad3 are functionally and structurally distinct, we aimed at addressing their individual roles through selective silencing of the expression of either Smad. We hypothesize that these Smads have differential roles in regulating cell fates to maintain stem cell homeostasis. By selectively silencing Smad2 or Smad3 expression using lentiviral shRNAs in hESCs and mEpiSCs under undifferentiated conditions, we found that Smad2, but not Smad3, was the main regulator of pluripotency. This correlated with the functional association of Smad2, but not Smad3, with regulatory sequences of the Nanog promoter, allowing TGF-beta/Activin signaling to induce Nanog upregulation through Smad2 in hESCs and mEpiSCs. Decreased Nanog expression, due to downregulation of Smad2 expression correlated with enhanced expression of early differentiation markers, such as Brachyury (Bra) (mesoendoderm) and Cdx2 (trophectoderm), and decreased expression of the pluripotency marker Oct4. Since these early differentiation genes are targets of BMP signaling, we examined the levels of autocrine BMP signaling in control and Smad2 silenced cells. Phosphorylation of Smad1, resulting from autocrine BMP signaling, was significantly higher in Smad2 silenced cells than in control cells. Furthermore, treatment

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of cells with Noggin, a BMP antagonist, suppressed the aberrant increase in Cdx2 and Bra expression, suggesting that the higher BMP responsiveness in Smad2 silenced cells enhances Cdx2 and Bra expression in hESC and mEpiSCs. Cdx2 and Oct4 are mutually antagonistic in mouse embryos and ES cells, and Nanog also plays a role in Oct4 in Cdx2 suppression. To further define the possible link between decreased Nanog expression and higher BMP responsiveness as a result of Smad2 silencing, we individually manipulated the expression levels of Cdx2, Nanog and Oct4 in control and Smad2 silenced cells. With downregulation of Nanog expression in control cells, we detected neither a higher BMP responsiveness nor a comparable increase in Cdx2 and Bra expression, as we found in Smad2 silenced cells. Cdx2 suppression in Smad2 silenced cells also did not recover Nanog expression as an immediate response, but resulted in rescue of Oct4 expression. Our data strongly suggest that the decrease in Nanog expression and the higher BMP responsiveness, observed in Smad2 silenced cells, occur independently yet affect pluripotency synergistically.

Poster Board Number: F-3009

INHIBITION OF EXTRACELLULAR E-CADHERIN IN HUMAN EMBRYONIC STEM CELLS

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The extracellular domain of E-cadherin molecules exhibit homophilic binding to adjacent cells and are central to cellular organisation and morphogenetic regulation. E-cadherin is expressed at high levels in undifferentiated embryonic stem (ES) cells and induced pluripotent stem (iPS) cells, and functions to increase reprogramming efficiency of iPS cells. However, recent advances show that the function of E-cadherin in ES cells is not limited to cellular adhesion and survival, but is also involved in regulation of the pluripotent state. Results from our laboratory show that loss of E-cadherin confers LIF-independent self renewal in mouse ES cells. Furthermore, E-cadherin null ES cells exhibit substantial transcript alterations compared to the parental line. However, the effect of loss of E-cadherin expression on global transcript expression in undifferentiated human ES cells has not been previously assessed. To examine this, gene expression profiles of human ES cells grown under defined, feeder-free culture conditions in the presence of a neutralising antibody/or peptide inhibitor of E-cadherin were analysed using an Affymetrix human genome DNA microarray. Differential gene expression (Q value ≤0.05 and fold change ≥1.5) between antibody-treated versus control IgG-treated cells revealed 400 transcript alterations, of which 84% were down regulated in response to E-cadherin inhibition. However, cells treated with peptide versus control resulted in 10-fold less differentially expressed genes. Of these 40 transcripts, only 14% were down regulated in E-cadherin abrogated cells. Comparative data shows that only 9 transcripts are conserved between antibody- and peptide-treated cells. Abrogation of E-cadherin in human ES cells results in loss of colony integrity and cells exhibit a mesenchymal-like phenotype. Furthermore, initial observations show that cells treated with an E-cadherin peptide antagonist retain expression of markers concomitant with undifferentiated ES cells(> 7days).

Poster Board Number: F-3010

THE ROLE OF SNON IN THE PLURIPOTENCY OF HUMAN EMBRYONIC STEM CELLS

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Activin/Nodal signaling is required to maintain pluripotency in human embryonic stem cells (hESCs) and to prevent these cells from differentiating down the neuroectoderm pathway. These essential functions are achieved through the phosphorylation and nuclear localisation of Smad2/3, which activates the expression of the core pluripotency factor NANOG. To further dissect the roles of this signaling pathway in hESCs we pharmacologically inhibited activin/nodal signaling in H9 cells using SB431542 and performed a microarray to identify genes with a significantly different expression from hESCs grown in activin/nodal. We identified SNON (SKI-like) as a gene that had a significantly lower expression following activin/nodal inhibition. SnoN has not previously been investigated in hESCs but has been reported to be an oncoprotein and a negative regulator of activin/nodal (TGFbeta) signaling in other cells types. We demonstrated that SnoN expression is activated by activin/nodal signaling in hESCs by qPCR and that Smad2/3 bound to the SnoN promoter by Chromatin Immunoprecipitation. Using Nanog, Oct4 and Sox2 overexpressing and knockdown hESC lines we demonstrated that Nanog regulates the expression of SnoN. Interestingly, we also demonstrated that SnoN interacts with Smad2/3 in hESCs and negatively regulates the transcriptional activity associated with Activin/Nodal pathway in hESCs. To further examine the role of SnoN in pluripotent stem cells, we performed functional studies by performing gain and loss of function experiments. Overexpression of SnoN led to a decrease in Nanog expression, consistent with the role of this factor in negatively regulating the activin/nodal pathway. Conversely, knockdown of SnoN expression, altered the morphology of hESCs when compared to controls without inducing markers of differentiation but while inducing change in expression of cell cycle regulators. We therefore propose that SnoN plays an important role in pluripotency both by acting as a negative regulator of the activin/nodal pathway in hESCs and by playing a role in allowing hESCs to exit the pluripotent state and to undergo differentiation.

Poster Board Number: F-3011

OXYGEN-REGULATED TRANSCRIPTIONAL NETWORKS CONTROLLING HUMAN EMBRYONIC STEM CELL PLURIPOTENCY

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The pluripotent human embryonic stem cells (hESC) are isolated from the inner cell mass (ICM) of blastocyst stage embryos. These embryos receive metabolites and oxygen from the uterine fluid containing 2% - 5% of dissolved oxygen. Early embryo develops in low oxygen environment suggesting that hypoxic growth conditions are physiological for hESC cultures. Indeed, hypoxic growth conditions (1% - 4% O2) reduce spontaneous differentiation and enhance hESC self-renewal indicating that the processes mediating spontaneous differentiation are suppressed under low oxygen. However, studies on how hypoxia influences pluripotency have

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proved contradictory. It is not understood how hypoxia contributes to pluripotency and self-renewal. To study the mechanisms involved in oxygen-regulated pluripotency and self-renewal, three different hES cell lines (HS401, H9 and HS360) were plated on Matrigel and cultured in 4% (hypoxia) or in ambient oxygen concentration for a range of fixed time intervals. Consistent with the hypothesis of hypoxia supporting pluripotency, our results revealed that in response to low oxygen environment, hES cells activate canonical hypoxia responses and inhibit the down regulation of the pluripotency markers. To identify the hES cell specific transcriptional programs regulated by oxygen, RNA extracted from all the time points was analysed by the Affymetrix Human Exon 1.0 ST array platform. When statistically ($p<0.01$) and biologically (fold change > 1.5) significant changes were taken into account nearly 200 upregulated and almost 100 downregulated hits were detected in hypoxic conditions. These results indicate that hESCs grown in the physiological environment of ICM cells have altered transcriptional profile which can have major effect on their behavior and differentiation potential.

Poster Board Number: F-3012

DERIVATION AND CLONAL SURVIVAL OF HUMAN EMBRYONIC STEM CELLS ON HUMAN LAMININ-521-BASED MATRIX IN XENO-FREE AND CHEMICALLY DEFINED ENVIRONMENT

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We have cloned, produced and purified as human recombinant proteins all laminin isoforms that are expressed in human embryonic stem (hES) cells, and studied their effects on cultured hES and human induced pluripotent stem (iPS) cells. Laminins-511 (LN-511) and -521 (LN-521) were able to support self-renewal of the cells under completely chemically defined and xeno-free conditions. Moreover, cell culture dish coating with LN-521 permitted survival and rapid expansion of dissociated cells into single cell suspensions hES and iPS cells without the use of Rho-associated kinase (ROCK) inhibitor. Expansion of pluripotent hES and hiPS cells on LN-521 closely resembles standard cell culture methods, such as culturing of fibroblasts, and allows 1:20-1:30 passaging ratios. After several months in culture, hES cells growing on LN-521 coatings: (i) expressed markers of pluripotency, Oct-4, Nanog, Sox-2, and SSEA-4, (ii) could be *in vivo* and in vitro differentiated into lineages of all three germ layers and, (iii) had normal karyotype. In contrast, dissociated cells did not survive on LN-111 and LN-121, and very poorly on LN-511. Clonal survival of hES cells on all four laminin coatings was low. On LN-521, cell survival was associated with rapid migration that was dependent on interaction with integrin α6β1. We have additionally developed a new human LN-521 -based coating matrix that for first time allows derivation of new hES cell lines under completely defined and xeno-free conditions. Importantly, the new matrix permitted clonal survival of hES cells without ROCK-inhibitor. Neither LN-521 nor other components of the new matrix alone allow clonal expansion of hES cells. The new human coating matrix in connection with xeno-free and chemically defined media is most useful for the development of human cells for regenerative medicine purposes.

Poster Board Number: F-3014

METABOLIC CHANGES IN HUMAN EMBRYONIC STEM CELLS ARE REGULATED BY FGF2

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Human embryonic stem cells (hESCs) are expected, thanks to their unique properties such as pluripotency and self-renewal, to become in future one of the sources of cells for therapeutic use. It has been known, that FGF2 maintains pluripotency in hESCs. While the effect of FGF2 on pluripotency signaling has been under careful investigation, not much is known about its effect on energy metabolism. The idea that hESCs rely preferentially on a "clean" glycolysis rather than on "dirty" oxidative phosphorylation matches the need of hESCs to maintain intact genome. It is also manifested by rather undeveloped, circular mitochondria with absent cristae in hESCs compared to their differentiated counterparts. In this study we have focused on defining the role of FGF2 in the switch between glycolysis based metabolism in hESCs and oxidative phosphorylation based metabolism in differentiated cells. By the use of chemical inhibitor dichloroacetate (DCA) we inhibited pyruvate dehydrogenase kinase (PDHK), leading to increased pyruvate flux into the mitochondria and thus shifting the metabolism of the cells from glycolysis toward the oxidative phosphorylation. Eight days of DCA treatment resulted in rapid decrease of the proliferation capacity of cells cultivated in the absence of FGF2, while the presence of FGF2 partially rescued the phenotype, enabling the cells to proliferate even under the conditions of limited glycolysis. Viability of the cells remained constant under all tested conditions making the difference only in the function of proliferative capacity. Although the DCA treatment significantly affected proliferation rate, these changes were not accompanied by a decrease of expression of undifferentiated stem cell markers NANOG and SSEA3, suggesting that it is not differentiation which enables the cells to survive in the presence of DCA, despite the fact that mitochondria undergo structural changes during the DCA treatment that are similar to changes observed during differentiation. Western blot analysis shows response of hexosa kinase, the key glycolysis-regulating enzyme to FGF2 suggesting that the pleiotropic effect of FGF2 inclused regulation of basic metabolic pathway. These results shed new light on the FGF2 function in metabolism. While it maintains the pluripotency status it can also support the growth of pluripotent cells under conditions favoring the oxidative phosphorylation.

Poster Board Number: F-3015

GLUCOSAMINE PROTECTS LONG-TERM HYPOXIA-INDUCED MOUSE EMBRYONIC STEM CELL APOPTOSIS THROUGH ATTENUATION OF ER STRESS: INVOLVEMENT OF SP1 GLYCOSYLATION AND HSP70 EXPRESSION

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Glucosamine (GlcN), a natural amino monosaccharide, is a metabolic precursor of O-linked N-acytylglucosamine (O-GlcNAc) which is essential for life in mammalian cells and involved in regulat-

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ing nuclear and cytoplasmic proteins glycosylation in a manner analogous to protein phosphorylation. However, effects of GlcN on hypoxia-induced apoptosis of mouse embryonic stem cells (mESCs) have not been reported. Thus, we examined the role of GlcN in protection of ER stress-induced mESC apoptosis under long-term hypoxia and its related signal pathways. In this study, hypoxia increased level of reactive oxygen species (ROS) and altered ER stress marker proteins expression [increased C/EBP-homologous protein (CHOP), and decreased in glucose-regulated protein 78 (GRP78)] in a time-dependent manner, which was blocked by pretreatment of GlcN. In addition, pretreatment of GlcN increased OGT (O-GlcNAc transferase) expression but decreased OGA (O-GlcNAcase), subsequently increased level of specificity protein 1 (SP1) glycosylation in hypoxia, which was blocked by pretreatment of ST 045849 (O-GlcNAc trasferase inhibitor) but not by PUGNAc (O-GlcNAcase inhibitor). GlcN-induced glycosylation of SP1 increased expression of heat shock protein 70 (HSP70), which was decreased by pretreatment ST 045849, OGT- , or SP1-specific siRNA, but not by PUGNAc. Furthermore, GlcN-induced protection of ER stress in hypoxia was blocked by SP1-, HSP70-specific siRNA, or VER155008 (HSP70 inhibitor). These results suggest that the GlcN-induced increase in HSP70 by O-GlcNAc-SP1 exerts protective effects on hypoxia-induced ER stress in mESCs. In hypoxic condition, GlcN decreased cleaved caspase-3 and increased in bcl-2 and cellular inhibitor of apoptosis proteins (c-IAPs), which were attenuated by pretreatment of SP1-, HSP70-specific siRNA, or VER155008. Finally, GlcN-related signal molecule specific-siRNAs and inhibitors blocked hypoxia-induced increase in DNA fragmentation and LDH release level and decrease in [^{3}H]-thymidine incorporation and cell number,. In conclusion, GlcN protected hypoxia-induced apoptosis of mESCs through attenuation of ER stress via SP1 glycosylation and HSP70 expression.

Poster Board Number: F-3016

A COMPARATIVE STUDY OF STANDARD AND NOVEL PROTOCOLS FOR MOUSE EMBRYONIC STEM CELL CULTURE

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Although the use of embryonic stem (ES) cells in regenerative medicine is still in an early phase, the number of stem cell research groups has expanded rapidly resulting in ES cells presently being one of the most intensively studied cell types. In recent years several new ES cell culture protocols have been published; nevertheless, most groups still rely on standard methods such as growing the cells on mouse embryonic fibroblast feeder cells or on gelatin in media supplemented with fetal bovine serum and leukemia inhibitory factor (LIF). However, these techniques have several drawbacks: 1) the need for feeder-cells (for feeder dependent cell lines), 2) undefined media and media containing animal derived components and 3) low level of spontaneous differentiation in the culture. In the present investigation we present a benchmarking study comparing the standard culture protocols with two newly described ones: 1) growing cells in semi-adherence in a media containing two small molecule inhibitors (CHIR99021, PD0325901) and; 2) growing cells in a spheroid suspension culture in a defined media containing LIF and bFGF. Two feeder-dependent mES cell lines (R1 and C57) and two cell lines adapted to feeder-independent growth (E14 and E14/T) were used in this study. The overall aim has not only been to compare self-renewal and differentiation capacity, but also ease-of-use and cost efficiency. Our data confirm that the recently described chemical approach of culturing mES cells with small molecule inhibitors allows for cultures with high proliferation rates and negligible spontaneous differentiation. Furthermore, we show that this media can, within a few passages, rescue and cleanup cultures that have started to deteriorate. Interestingly, although this novel culturing condition can be readily used for feeder-dependent as well as suspension growth, the cells do not adhere to standard polystyrene cell culture vessels or to common coating substrates such as fibronectin, vitronectin, collagen or gelatin. However, we show that addition of low levels of serum added to this media promptly triggers cells to adhere to most surfaces. In fact, this allows for a quick and effective adaption of feeder-dependent mES cell lines to be maintained on a feeder-free surface.

Poster Board Number: F-3017

MULTIPLE COMPLEX MECHANISMS REGULATE INDIVIDUAL PROTEIN LEVELS IN MOUSE ES CELLS

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Mouse embryonic stem cells predominantly utilize homologous recombination (HR) to repair DNA double strand breaks. This is in contrast to somatic cells which predominantly utilize non-homologous end joining (NHEJ). The proteins involved in HR repair, but not NHEJ, are elevated many fold in ES cells compared with mouse embryo fibroblasts (MEFs). To gain insight into the mechanisms that regulate protein levels in ES cells, we have investigated how the protein level of Rad51, a key component of HR, is controlled in MEFs and ES cells. The level of Rad51 is about ~15-fold higher in ES cells than in MEFs whereas the level of its mRNA is only ~2-fold higher. These data indicate that the difference in Rad51 mRNA levels due to differences in transcription rates or mRNA stability are insufficient to account for the large disparity in protein levels. Comparison of Rad51 protein half lives between ES cells and MEFs also did not explain the elevated level of Rad51 protein in ES cells. When translation rates of Rad51 were assessed, however, the data showed that Rad51 was translated with much greater efficacy in ES cells than in MEFs. To determine whether this high level of translation in ES cells is a general ES cell phenomenon or is a characteristic of proteins involved with recombination and cell cycle progression, we compared the mechanisms that regulate the level of Pcna. The protein half-lives and rates of synthesis of Pcna and Rad51 within ES cells and between ES cells and MEFs were quite different, demonstrating that regulation of Rad51 does not reflect the mechanisms by which the levels of most proteins are maintained in ES cells. Similarly, these mechanisms cannot be generalized to those involved in DNA replication and cell cycle control. Finally, we show that only a small proportion of the abundant Rad51 protein population in ES cells is activated under basal conditions and recruited to DNA DSBs when damage occurs. These data suggest that mouse ES cells are poised to rapidly respond to DNA damage.

Poster Board Number: F-3018

PORCINE EMBRYONIC AND INDUCED PLURIPOTENT STEM CELLS ANALOGOUS TO LIF-DEPENDENT, NAÏVE EMBRYONIC STEM CELLS OF THE MOUSE

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Authentic embryonic stem cells (ESC) may never have been successfully derived from the inner cell mass (ICM) of pig and other

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ungulates, despite over 25 years of effort. Recently, porcine induced pluripotent stem cells (piPSC) were generated by reprogramming somatic cells with a combination of four factors OCT4, SOX2, KLF4 and c-MYC (OSKM) delivered by lentiviral transduction. The established piPSC are analogous to FGF2- dependent human (h) ESC and murine "epiblast stem cells," and are likely to advance swine as a model in biomedical research. Here, we report for the first time, the establishment of LIF-dependent, so called naïve type pluripotent stem cells (1) from the inner cell mass (ICM) of porcine blastocysts by up-regulating the expression of KLF4 and POU5F1; and (2) from umbilical cord mesenchyme (Wharton's jelly) by transduction with OSKM factors and subsequent culture in the presence of LIF-based medium with inhibitors that substitute for low endogenous expression of c-MYC and KLF4 and promote pluripotency. The 2 compounds that have been used in this study are, CHIR99021 (CH), which substitutes c-MYC by inhibiting GSK3B and activating WNT signalling and Kenpaullone (KP), which inhibits both GSK3B and CDK1 and supplants KLF4 function. The lentiviral vectors employed for introducing the re-programming genes were modified for doxycycline-mediated induction of expression (tet-on) and are 'floxed' for Cre-mediated recombination and removal of transgenes following complete reprogramming. Two LIF-dependent cell lines have been derived from the ICM cells of late d 5.5 *in vitro* produced blastocysts and four from umbilical cord mesenchyme recovered from fetuses at d 35 of pregnancy. The derived stem cell lines are alkaline phosphatase-positive, resemble mouse embryonic stem cells in colony morphology, cell cycle interval, transcriptome profile and expression of pluripotent markers, such as POU5F1, SOX2 and surface marker SSEA1. They are dependent on LIF signalling for maintenance of pluripotency, can be cultured over extended passage (50) with no senescence. Of importance, the ICM-derived lines have been successful in their ability to form teratomas. The cells could be cultured in feeder free conditions on a laminin-coated matrix in the presence of chemically defined (N2B27) medium and can be coaxed to differentiate under xeno-free conditions. Currently, the piPSC lines are being investigated for their ability to give rise to teratomas and to produce a live offspring by nuclear transfer.

Poster Board Number: F-3019

DIFFERENTIATION REVERSIBILITY BETWEEN MOUSE EMBRYONIC STEM CELLS AND EPIBLAST STEM CELLS

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Recently, a different type of pluripotent stem cells derived from the mouse epiblast of post-implantation embryos, termed "mouse epiblast stem cells" (mEpiSCs) was established. These stem cells have different characteristics (i.e. morphology, mechanism of maintenance of undifferentiated state and differentiation potency) from mouse ESCs. The differentiation capability of mEpiSCs indicates that mEpiSCs are less pluripotent than mESCs and may reflect a developmental stage gap with mESCs and recipient embryos. A major focus of the study on mEpiSCs is the resemblance between mEpiSCs and conventional human ESCs (hESCs). Interestingly, although they are derived using blastocysts, conventional hESCs seem to correspond very closely to mEpiSCs with respect to many characteristics. Understanding and elucidating the mechanisms/factors involved in the differentiation may enable us to switch these two pluripotent states, i.e. the shuttle between mESC- and mEpiSC-states can lead to increasing the value of the hESCs. For example, it may provide a basis for generating more ideal types of mESC-like human pluripotent cells from conventional hESCs. However, the factors that affect the pluripotent state conversions have not been fully elucidated. In this study, we tried to induce conversion of the pluripotent state between mESC- and mEpiSC-state. To visualize the pluripotent state conversion, we used Oct4-dePE GFP marker. The analysis was performed by Realtime-PCR, Westernblot, Immunofluorescence and production of chimeric mice. Firstly, we induced mEpiSCs from mESCs by changing the culture condition, which is hypoxic environment. The converted cells were obtained as SSEA-1+/ Oct4-dePE GFP+ fraction, these cells expressed epiblast markers and, on the contrary, in which expression of ICM specific marker genes were declined. Next, to convert from mEpiSCs to mESCs, we tried the induction by using small molecules which would enable the activation of the beta catenin pathway that is one of the pathways for maintaining undifferentiated state in mESCs. These induced cells expressed ES cell-specific gene such as *Klf4* and *Stra8* and downregulated markers of lineage specification. In both cases, we succeeded in the conversions of pluripotent state. The present study provides evidence that oxygen concentration and/or activation of beta catenin signaling pathway are important factors for the promotion and stabilization of the mESC- or mEpiSC-state. Determination of the factors and signal cascade enhancing the conversion or increasing mESC- or mEpiSC-state stabilities in future studies are essential to correctly understanding the developmental stages and properties of pluripotent stem cells, which may lead to a more ideal model system for studying human development.

Poster Board Number: F-3020

AMD1 IS ESSENTIAL FOR MOUSE EMBRYONIC STEM CELL SELF-RENEWAL AND IS TRANSLATIONALLY REGULATED BY MIR-762.

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The gene expression networks governing embryonic stem cell pluripotency are complex and finely regulated during differentiation towards specific lineages. We describe a new role for Amd1, a key enzyme in the polyamine synthesis pathway, in regulating mouse ES cell self-renewal and differentiation to the neural lineage. Amd1 is highly expressed in ES cells and is down regulated in Neural Precursor Cells (NPCs). We show that an NPC enriched miRNA targets the Amd1 3'UTR for translational repression during NPC differentiation and that over-expression of Amd1 blocks ESC to NPC conversion. In addition, we demonstrate that high levels of Amd1 are required for maintenance of the ES cell state and forced over-expression of Amd1 can delay ES cell differentiation on removal of LIF. We will present data demonstrating that Amd1 is required to promote high levels of Myc to maintain the ES cell state. We propose that Amd1 plays an essential role in the regulation of polyamine levels in ES cells and its translational down regulation is required for neural differentiation.

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Poster Board Number: F-3021

LIF INDUCED STAT3 SIGNALING SUPPRESSES FGF1 INDUCED ERK1/2 ACTIVATION TO INHIBIT THE DOWNSTREAM DIFFERENTIATION IN MOUSE EMBRYONIC STEM CELLS

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In regular culture condition with leukemia inhibitory factor (LIF), majority of mouse embryonic stem cells (mESCs) were maintained in a self-renewal stage; very few mESCs were shown in differentiated morphology. The mESCs tend to differentiate when LIF was withdrawn, and the differentiation process could be enhanced when exogenous FGF was introduced. Here, we show that, even in the presence of exogenous FGF1, mESCs could still maintain self-renewal and expression of pluripotency markers when LIF was present. To elucidate the mechanism in which LIF dominates over FGF1, extracellular signal-regulated kinase 1/2 (Erk1/2) signaling of 46C mESCs cultured in medium containing FGF1 or LIF/FGF1 was examined. The results demonstrated Erk1/2 was activated by FGF1 in the absence of LIF, but the FGF1-induced Erk1/2 phosphorylation would be suppressed when LIF was introduced. Moreover, the FGF1-Erk1/2 down regulation was inhibited by signal transducer and activator of transcription 3 (Stat3) inhibitor WP1066, suggesting that LIF-induced Stat3 activation plays an important role in FGF1-Erk1/2 inhibition in 46C mESCs. We further demonstrated that the binding affinity of phospho-Erk1/2 and Sprouty2 was increased via Stat3 activation. Binding of phospho-Erk1/2 and Sprouty2 blocks the activation of Erk1/2 signaling, thus inhibits the downstream differentiation process in 46C mESCs. Our finding demonstrates, for the first time, that LIF-induced Stat3 phosphorylation plays an important role in promoting the binding of phospho-Erk1/2 and Sprouty2, and thus inhibits the FGF-induced differentiation.

Poster Board Number: F-3022

TBX3 CENTERED REGULATORY NETWORK IN THE SELF -RENEWAL AND PLURIPOTENCY OF MOUSE EMBRYONIC STEM CELLS

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Tbx3 is a member of the T- box transcription factor family that plays key roles during mouse development. Tbx3 is the only family member that is highly expressed in the inner cell mass. Previous work from our own and other laboratories has demonstrated that Tbx3 is essential for the maintenance of the mouse embryonic stem (mES) cell self-renewing pluripotent state. Tbx3 has also been shown to play an important role during induced pluripotent stem (iPS) cell reprogramming. RNAi-mediated down regulation of Tbx3 in mES cells changed the expression profile of a specific set of more than 100 genes. No changes in this expression profile were observed upon down regulation of Oct4, Nanog or Sox2 and this was found to be statistically significant. In addition, previous protein-protein interaction studies centered on Oct4 and Nanog did not identify Tbx3 as an interaction partner. We therefore hypothesized that Tbx3 regulates self-renewal and pluripotency by mechanisms largely distinct from the canonical Oct4/Nanog/Sox2/Tcf3 axis. To study the role of Tbx3 within the mES cells we used an affinity pull down strategy to identify protein-protein interaction partners and genome-wide binding targets of this factor in mES cells using IP-LC/MS-MS and ChIP-seq methodology. We found that Tbx3 binds to the genomic regions of ES cell regulatory genes such as Oct4, Nanog, Sox2, Esrrb, Dppa3 and Sall4. It also binds to genomic loci involved in mesodermal (*Myo1b, T*) and ectodermal (*Neurog1, Neurog3*) development. We identified peptides from more than 100 Tbx3-interacting proteins by IP-LC/MS-MS analysis. This protein-protein interaction network contains a number of gene-products that were not found to interact with other regulatory molecules such as Oct4, Nanog, Dax1, Esrrb, etc. This group of proteins is involved in the regulation of transcription, chromatin binding and mRNA processing pathways. Using RNAi knock down we found that some of these proteins are required for the maintenance of mES self-renewal. In addition, many Tbx3 interaction partners are bound by Tbx3 in ChIP-Seq analysis and are highly expressed in ES cells and/or iPS cells compared to differentiated cells. In summary, we have constructed a transcriptional regulatory network of Tbx3 protein-protein and protein-DNA interactions in ES cells. We will integrate these data with the known protein-protein and protein-DNA mES cell regulatory networks to better understand the global control of self-renewal and pluripotency.

Poster Board Number: F-3023

ARTIFICIAL CELLULAR MICROENVIRONMENTS USING ELECTROSPUN SYNTHETIC NANOFIBERS MAINTAIN SELF-RENEWAL OF MOUSE EMBRYONIC STEM CELLS

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Pluripotent stem cells, including embryonic and induced pluripotent stem (ES and iPS) cells, have a great potential for applications in cell-based therapies and regenerative medicine with their unique properties to proliferate indefinitely and to differentiate into all types of cells in a body. However, there are some concerns about conventional culturing methods involving feeder cells and serum replacement. Furthermore, conventional methods do not take into account the effects of cellular microenvironments appropriately, although these microenvironments have been found to play an important role in regulating stem cell fate decision *in vivo*. It is still challenging to create such microenvironments in vitro. Even though the leukemia inhibitory factor (LIF) was identified as a critical factor to maintain mouse ESC self-renewal, other mechanisms of self-renewal that are more closely related to the microenvironmental regulation remain to be determined. Nanofibers are advantageous for stem cell research as they can mimic *in vivo* cellular microenvironments. Recently, some reports have described their applications in stem cell expansion and differentiation as well as transplantation. In comparison with a conventional flat substrate, nanofibers allow controlling over the bulk-porosity, the surface texture and the three-dimensional (3D) surface topology for cell adhesion at a nanometer scale, altering focal-adhesion signalling cascades, and as a result, the stem cell fate. In addition to the control of 3D topological features, nanofibers can also be functionalized with bioactive molecules to further enhance controllability of cell growth factors and cell-cell interactions. Moreover, nanofibers made of biodegradable materials allow the process of stem cell differentiation to be precisely controlled during tissue engineering and cell-based transplantation therapy. In the past

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few years, we created an artificial cellular microenvironmental cue by using a synthetic polymer, polymethylglutarimide (PMGI), as the nanofiber material, because of its biocompatibility and cost effectiveness. In this study, we utilized PMGI-based nanofibers as a cellular scaffold for maintaining mESC self-renewal in feeder-free conditions. We found that mESC adhesion was depended on the density of nanofibers, and the high-density of nanofibers facilitated mESC adhesion on their surface compared with lower density of nanofibers. Interestingly, since mESC couldn't adhere on the surface of a PMGI-coated flat substrate, these results indicated that nanofiberous structures were very important for facilitating mESC adhesion on substrates. In addition, mESCs cultured on the high density of nanofibers were able to form their colonies with a three-dimensional semi-spherical shape during culture, which were identical with the conventional mESC culture setting. In terms of mESC proliferation, mESCs on nanofibers have similar growth rates with those of a setting of conventional co-culturing system with feeder cells. Finally, we evaluated the pluripotent status of mESCs cultured on nanofibers by immunocytochemistry, flow cytometry, RT-PCR and embryoid formation assays. According such multiple angles of evaluation, we concluded that mESCs on nanofibers maintain their pluripotent status for the over time. In the future, our goal is to develop a three-dimensional architecture of a cellular substrate for controlling human ES/iPS cells.

Poster Board Number: F-3024

INVOLVEMENT OF A NOVEL PREIMPLANTATION-SPECIFIC GENE ENCODING THE HIGH MOBILITY GROUP BOX PROTEIN HMGPI IN EARLY EMBRYONIC DEVELOPMENT AND DERIVATION OF MOUSE EMBRYONIC STEM CELLS

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Mining gene-expression-profiling data identified a novel gene that is specifically expressed in preimplantation embryos. Hmgpi, a putative chromosomal protein with two high-mobility-group boxes, is zygotically transcribed during zygotic genome activation, but is not transcribed postimplantation. The Hmgpi-encoded protein (HMGPI), first detected at the 4-cell stage, remains highly expressed in pre-implantation embryos. Indeed, Hmgpi was also expressed in blastocyst outgrowth and embryonic stem (ES) cells through derivation of ES cells. Interestingly, HMGPI is expressed in both the inner cell mass (ICM) and the trophectoderm, and translocated from cytoplasm to nuclei at the blastocyst stage, indicating differential spatial requirements before and after the blastocyst stage through derivation of ES cells. siRNA (siHmgpi)-induced reduction of Hmgpi transcript levels caused developmental loss of preimplantation embryos and implantation failures. Furthermore, reduction of Hmgpi prevented blastocyst outgrowth leading to generation of ES cells. The siHmgpi-injected embryos also lost ICM and trophectoderm integrity, demarcated by reduced expressions of Oct4, Nanog and Cdx2. The findings implicated an important role for Hmgpi at the earliest stages of mammalian embryonic development and derivation of ES cells. Furthermore, the Hmgpi gene is highly expressed in ES cells, but not in EC cells; Hmgpi is thus eligible as a putative ECAT (ES cell-associated transcript), whose ESTs are overrepresented in cDNA libraries from ES cells compared with those from somatic tissues and other cell lines including EC cells. It is also likely that Hmgpi is expressed in iPS cells, based on in silico analyses of expression profiles. Thus, Hmgpi is likely to have a role in maintaining pluripotent cells, since the ECATs such as Nanog, Eras and Gdf3 are required for pluripotency and proliferation of ES cells. In summary, Hmgpi is required early on in mammalian development to generate healthy blastocysts that implant successfully and produce ES cells. HMGPI translocates into the nucleus from cytoplasm at the blastocyst stage, which is importantly a turning point of early embryonic development when DNA-methylation levels are at their lowest and implantation takes place. The nuclear HMGPI in blastocysts and ES cells is expected to act as a transcription factor to regulate gene expression networks underlying the generation, selfrenewal and maintenance of pluripotent cells. Because E7 embryos have already stopped expressing Hmgpi, it is likely that Hmgpi stage-specifically regulates a set of genes that drive peri-implantation development. It will be valuable to identify both cofactors that bind HMGPI and recognize specific DNA sequences, as well as genes that are regulated by Hmgpi using ES cells. A better understanding of the Hmgpi transcriptional network will also improve culture methods for healthy blastocysts and for generating, maintaining and differentiating ES cells.

Poster Board Number: F-3025

HIGH-THROUGHPUT FUNCTIONAL IDENTIFICATION OF ACTIVE DNA REGULATORY ELEMENTS IN MOUSE EMBRYONIC STEM CELLS.

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Mammalian ES cells represent a unique cell state characterized by the properties of self-renewal and pluripotency. The distinguishing properties of ES cells, and those of their differentiated derivatives, are largely governed by different sets of core Transcription Factors (TFs) that serve to establish cell-specific chromatin landscapes and hierarchies of gene expression. Thus a full understanding of these cell states, and the transitions between them, requires the identification of the target DNA elements and TFs comprising their distinct transcriptional circuitries. While the majority of analyses have focused on ChIP-based binding studies of Oct4, Sox2, and Nanog, additional factors contributing to pluripotency remain largely uncharacterized, and little is known regarding the TFs and target DNAs mediating the earliest events of lineage specification of ES cells. To address this, we have developed a new approach allowing the functional isolation of active stage-specific promoter and enhancer elements from ES cells and ES cells undergoing differentiation. Based on the classic observation that active promoter and enhancer elements lie within nucleosome-free regions (NFRs) within cellular chromatin, we first devised a simple method for isolating short (~150bp) NFR-DNAs from ES cell chromatin. Lentiviral libraries in which individual ES-derived NFRs drive expression of a GFP reporter gene were constructed and used to isolate NFR DNAs exhibiting differential activation of GFP in transduced ES- and differentiated cells. Preliminary data correlating the genomic loci of the isolated elements with gene expression data and local histone modifications are consistent with the notion that these NFR-DNAs are likely to represent transcriptional regulatory modules with stage-specific activity. Current efforts entail the High-throughput (Illumina) sequencing and global mapping of these functionally-identified DNAs to their positions within the mouse genome. These data will permit the addition of functional information to the annotation of the mouse genome loci that can be integrated with other highthrouput mapping data, and will facilitate the development of

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robust models of ES transcriptional networks and the identification of novel, stage-specific TFs.

Poster Board Number: F-3026

THE ROLE OF ZFX IN THE INDUCTION AND SELF-RENEWAL OF THE MOUSE EMBRYONIC STEM CELL STATE

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The generation of induced pluripotent stem cells (iPSCs) by factor-based reprogramming harbors immense potential as it circumvents ethical and medical issues faced by embryonic stem cell (ESC) applications. However, despite the rapid advancement in the field of reprogramming, iPSC derivation has neither been fully optimized nor understood. Thus, we investigated whether several transcription factors involved in mouse ESC and iPSC biology possess functions which enhance reprogramming. Herein we identified the zinc finger transcription factor Zfx as a novel enhancer of both the efficiency and kinetics of iPSC derivation. The knockdown of Zfx revealed that it is indeed important for the self-renewal of mouse ESC state. In-depth analysis of gene expression changes and chromatin binding data uncovered the transcriptional network governed by Zfx. We showed that Zfx binds to and regulates a wide variety of gene classes, particularly those with roles in gene expression, ESC self-renewal, proliferation, as well as those which are implicated in cancer. Our direct target analysis revealed that Zfx controls a unique ESC subprogram, ensuring the maintenance of ESC self-renewal, viability, and proliferation while protecting against malignancy.

Poster Board Number: F-3027

UNLIMITED SELF-RENEWAL OF MOUSE ES CELLS WITHOUT MYC/MAX COMPLEXES UNDER 2I CONDITION

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Myc family proteins comprising c-Myc, N-Myc and L-Myc have been shown to be crucial for preserving defining features of mouse ESCs under empirical culture conditions using LIF and serum. However, c-Myc expression levels in inner cell mass cells of blastocyst and ESCs cultured under 2i conditions using MAPK and GSK3β inhibitors are extremely low. To examine the role of Myc activity in ESCs in different culture conditions as well as in conventional condition, we generated ESCs lacking Max gene which encodes apparently obligate partner protein of all Myc proteins for exerting their almost all biological activities. In consistent with previous reports, we found that Max-null ESCs under empirical condition lose their pluripotent state abruptly and our data suggest that this loss of pluripotency is largely due to abnormal MAPK activation. We also found that Max-null ESCs show extensive cell death and these two major phenomena of Max-null ESCs do not occur randomly, but occur in a compulsory order with the loss of pluripotency marker gene expression occurring first, indicating that ESCs retaining principal pluripotency core proteins are rather refractory to execute cell death program. Our data also demonstrated that Myc activity can be eliminated without affecting pluripotent state if ESCs are under 2i condition. Thus, our data suggest that, unlike Oct3/4, Myc is not an absolutely required factor for preserving ESC status, but its requirement is rather restricted to ESCs cultured under empirical conditions without using MAPK inhibitor. We will also provide information of molecules responsible for detrimental phenotypes of Max-null ESCs under conventional condition.

Poster Board Number: F-3028

INTRERACTION BETWEEN PROFILIN-1/COFILIN-1 AND F-ACTIN THROUGH FAK- AND C-SRC/EGFR TRANSACTIVATION-DEPENDENT N-WASP/CDC42/TOCA-1 COMPLEX FORMATION IN E2-BSA-INDUCED MOUSE EMBRYONIC STEM CELL MOTILITY

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Long-term estrogen actions are vital for driving cell growth, but more recent evidence suggests that estrogen mediates more rapid cellular effects. However, the function of estradiol-17β (E2)-bovine serum albumin (BSA) in mouse embryonic stem cells (ESCs) has not been reported. Therefore, we examined the role of E2-BSA in mouse ESC motility and its related signal pathways. E2-BSA (10-8 M) significantly increased motility after 24 h incubation and increased filamentous (F)-actin expression; these effects were inhibited by the estrogen receptor (ER) antagonist ICI 182,780, indicating that E2-BSA bound membrane ERs and initiated a signal. E2-BSA increased c-Src and focal adhesion kinase (FAK) phosphorylation, which was attenuated by ICI 182,780. The E2-BSA-induced increase in epidermal growth factor receptor (EGFR) phosphorylation was inhibited by Src inhibitor PP2. As a downstream signal molecule, E2-BSA activated cdc42 and increased formation of a complex with the neural Wiskott-Aldrich syndrome protein (N-WASP)/cdc42/transducer of cdc42-dependent actin assembly (TOCA-1) which was inhibited by FAK siRNA and EGFR inhibitor AG 1478. In addition, E2-BSA increased profilin-1 expression and cofilin-1 phosphorylation, which was blocked by cdc42 siRNA. Subsequently, E2-BSA-induced an increase in F-actin expression, and cell motility was inhibited by each signal pathway-related siRNA molecule or inhibitors but not by cofilin-1 siRNA. A combined treatment of cofilin-1 siRNA and E2-BSA increased F-actin expression and cell motility more than that of E2-BSA alone. These data demonstrate that E2-BSA stimulated motility and contributed to the maintenance of undifferentiated state by interacting with profilin-1/cofilin-1 and F-actin through FAK- and c-Src/EGFR transactivation-dependent N-WASP/cdc42/TOCA-1 complex. Keywords: mouse embryonic stem cells, estradiol-17β, F-actin, profilin-1/cofilin-1, N-WASP/cdc42/TOCA-1 complex, cell motility

Poster Board Number: F-3029

AUTONOMOUS MOUSE EMBRYONIC STEM CELLS AND REPROGRAMMING EFFICIENCY - PROBING THE ROLE OF STOCHASTICITY IN A MINIMAL MOUSE STEM CELL ARCHITECTURE

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Embryonic stem cells (ESC) have the capacity to self-renew , remain pluripotent and provide a source of a variety of differentiated cell types. In stem cell biology and regenerative medicine it is important to understand what governs the ESC properties at

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the molecular level. Elucidating the interactions at this level in the context of reprogramming somatic cells into induced pluripotent stem (iPS) cells is also of relevance. It is a continual endeavor to understand the nature of the pluripotent state or "ground state", in which an ESC exhibits gene expression of numerous key stem cell regulators. Several molecular characterizations of the ESC pluripotent state were put forward both on the transcription factor (TF) and epigenetic levels. Whereas many players have been identified to be involved, it is desirable, to map out a core network that provides an autonomous system hosting the observed heterogeneity and reprogramming properties. Towards this goal, we propose a simplified computational model of a mouse embryonic stem cell network, in which a core of TFs (OCT4, SOX2, NANOG, and FGF4) interact both with themselves as well as being induced by external factors. Stochastic network simulations recapitulate the observed heterogeneity in NANOG, which is entirely driven by internal noise, and provides a framework to understand how the addition of external factors (LIF and 2i/3i media) play a role in modulating heterogeneity and allow cells to transit from one state to another. An important result is that the decision of staying in the ground state or committing to differentiate is stochastic and can be taken independently of the extrinsic stimuli. Furthermore, it is shown how the network, when in committed state, can be reprogrammed into iPS (ESC like) state by over-expressing OCT4. To this end, the model is extended to host the epigenetic role of OCT4, i.e. it "opens" up key pluripotent genes, among others Nanog. In the reprogramming context, the proposed model recapitulates the experimental result that reprogramming efficiency reaches higher levels when OCT4 is over-expressed within a specific concentration window. Our model suggests that choosing the iPS cell medium along with setting the degrees of over-expression should be considered for optimizing reprogramming efficiency. We have demonstrated how a stochastic computational model based upon a compact set of TFs with internal noise only, can account for the observed heterogeneity in the murine ESC population, characterizes the ground state under 2i/3i media conditions and describes transitions from and towards the ground state. The decision of remaining in the ground state or to commit can be taken independently of extrinsic stimuli. The model proposed here hosts the reprogramming process from fibroblast cells and provides an understanding of reprogramming efficiency as a function of OCT4 over-expression and the iPS medium considered.

Poster Board Number: F-3030

A PLURIPOTENCY FACTOR INTERACTS WITH NFAT AND ACTS AS A REPRESSOR OF NFAT-MEDIATED TRANSCRIPTION IN MOUSE EMBRYONIC STEM CELLS

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The calcineurin/NFAT signaling pathway is known to orchestrate diverse cellular and developmental programs. Our previous study indicates that the calcineurin/NFAT pathway is one of the essential pathways triggering the transition of mouse embryonic stem cells (mESCs) from the self-renewal state into early differentiation. However, the molecular mechanism by which the calcineurin/NFAT pathway is regulated in mESCs remains unknown. In this study, we have find that the expression and function of NFATs are under the control of pluripotency-associated factors, such as Oct4, Sox2 and Nanog. In particular, we discover that one of pluripotency-associated factor can abrogate the differentiation phenotype induced by over expression of NFATc3 in mESCs. At a molecular level, we show that the pluripotency factor interacts with NFAT proteins both in vitro and *in vivo*. Moreover, it suppresses NFAT transcriptional activities directly. Thus, our finding establishes a direct molecular and functional link between a pluripotency-associated factor and an important ESC differentiation-inducing pathway and will help us comprehend the mechanisms underlying ESC unique properties of self-renewal and pluripotency.

Poster Board Number: F-3031

C_{16}-CERAMIDE STIMULATES MOUSE EMBRYONIC STEM CELL MIGRATION THROUGH N-WASP/CDC42/ARP2/3 COMPLEX, COFILIN-1, AND A-ACTININ-1/-4-MEDIATED F-ACTIN REGULATION

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Recent studies reported that ceramide, major structural elements of cellular membrane, is a key regulatory factor in various cells behaviors and that its effect depends on the ceramide-induced association of specific proteins. However, molecular mechanisms that regulate ceramide-induced embryonic stem cells (ESCs) migration are still not well understood. Thus, we investigated the effect of ceramide on migration and its related signal pathways in mouse ESCs. Among ceramide isoforms with different fatty acid chain lengths (C_2-Cer, C_8-Cer, C_{16}-Cer, and $C_{18:1}$-Cer), C_{16}-cer greatly increased migration of mouse ESCs in a dose- (≥1 μM) and time-dependent (≥8[[Unsupported Character -]]h) manners, as determined by boyden chamber migration assay. C_{16}-Cer (10 μM) increased protein-kinase C (PKC) phosphorylation. Subsequently, C_{16}-Cer increased focal adhesion kinase (FAK) and paxillin phosphorylation, which were inhibited by PKC inhibitor bisindolylmaleimide I (1 μM). In experiments to determine downstream signaling molecules, C_{16}-Cer activated small G protein (cdc42), increased formations of complex with Neural Wiskott-Aldrich Syndrome Protein (N-WASP)/cdc42/Actin-Related Protein 2/3 (Arp2/3). This complex formation was disrupted by FAK and paxillin specific siRNA. Furthermore, C_{16}-Cer-induced increase of F-actin expression was inhibited by cdc42-, N-WASP-, or Arp2/3-specific siRNA, respectively. On the other hand, C_{16}-Cer induced F-actin regulation. Indeed, C_{16}-Cer decreased cofilin-1/F-actin complex formation via cofilin-1 phosphorylation, which was reversed by cdc42-specific siRNA. C_{16}-Cer also increased F-actin/α-actinin-1 or α-actinin-4 interaction in plasma membrane which was blocked by cdc42-specific siRNA. Finally, C_{16}-Cer-induced increase of cell migration was inhibited by each signal pathway related molecules siRNA or inhibitors, respectively. Further studies on the possible additional effects of ceramide on ESCs should aid in the understanding of their role in ESCs behaviors as well as further specific signaling mechanisms involved in the control of these functions. In conclusion, C_{16}-Cer increased mouse ESCs migration through regulation of PKC and FAK/paxillin-dependent N-WASP/cdc42/Arp2/3 complex formation as well as interaction between cofilin or α-actinin-1/-4 and F-actin.

Poster Board Number: F-3032

CELLULAR PHENOTYPING OF HOMOZYGOUS MOUSE ES CELLS.

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The exploitation of mouse embryonic stem (ES) cells to elucidate a variety of different areas in biology is currently under utilised

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and has great potential for high impact science. ES cells represent a genetically tractable model system to study basic cell biological and developmental processes on a genome-wide scale and will add an important new dimension to the functional annotation of mammalian genes. Large-scale genetic screens in cultured cells are presently hampered by the challenges of generating homozygous mutant cells. Moreover, genes essential for cell viability and growth are not easily recovered in loss-of-function screens. Therefore, inducible gene ablation strategies are required. Taking advantage of the EUCOMM and KOMP libraries of conditional targeting vectors, we have developed methods for the generation of conditional homozygous mutations in mouse ES cells (Tate & Skarnes, 2011). Elimination of gene function is dependent on the activation of ligand-inducible Cre recombinase in cells following treatment with tamoxifen. Thus, the effect of gene ablation can be assayed in undifferentiated ES cells or at any time following differentiation. We have further optimized the strategy to enable genetic screens in ES cells at scale. We are currently applying this approach to defining the role of chromatin associated proteins in various aspects of stem cell pluripotency. Here, we demonstrate the efficacy of gene ablation both in stem cells and differentiated derivatives and present examples from our screen.

Poster Board Number: F-3033

SPHINGOSINE-1-PHOSPHATE-INDCUDED FLK-1 TRASNACTIVATION STIMULATES G1/S TRANSITION OF MOUSE EMBRYONIC STEM CELLS VIA S1P1/3-DEPENDENT BETA-ARRESTIN/C-SRC PATHWAYS

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Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid metabolite that exerts important effects on numerous cellular events such as differentiation, migration, and proliferation via G-protein coupled receptors S1P1-5 in various cells. In addition, it has been known that several G protein-coupled receptor ligands transactivate cytokine receptors such as EGFR and PDGF. However, the effects of S1P signaling on mouse embryonic stem (mES) cells and whether S1P stimulates fetal liver kinase-1/kinase-insert domain-containing receptor (Flk-1/KDR), one of VEGF receptors, transactivation are not well understood. To address this issue, we examined the interaction between S1P receptor and Flk-1 and its role on mES cells proliferation. We observed that the S1P receptor 1-5 mRNA were expressed in mES cells and S1P treatment significantly increased S1P receptors 1-3 mRNA expression, which were supported by western blot analysis. In addition, S1P increased expressions of cell cycle regulatory proteins, [^{3}H]thymidine incorporation, and proliferation index as well as total cell number in a dose- and time-dependent manner. S1P 1-3 and Flk-1 were co-localized in lipid raft/caveolae microdomain and methyl-β-cyclodextrin (lipid raft disruptor) blocked S1P-induced mES cells proliferation. And S1P-induced proliferation were inhibited by pertussis toxin (Ptx; G protein inhibitor), VPC23019 (S1P 1/3 receptor antagonist), or W146 (S1P 1 receptor antagonist) but not by JTE013 (S1P 2 receptor antagonist), suggesting that the action of S1P mediated by S1P 1 and 3 receptors. Furthermore, S1P or VEGF treatment increased Flk-1 phosphorylation and S1P increased VEGF expression and secretion. Moreover, S1P elicited translocation of β-arrestin from cytosol/nuclear to membrane and increased level of co-immunoprecipitation with S1P1/3, which resulted in phosphorylation of c-Src. S1P-induced phosphorylation of Flk-1 was blocked by MβCD, Ptx, VPC23019, W146, β-arrestin specific siRNA, and PP2 (c-Src inhibitor), but not by VEGF neutralizing antibody or VEGF siRNA. In addition, S1P increased level of co-immunoprecipitation of S1P1/3 receptors with Flk-1, and expression of Flk-1 in lipid raft faction. Pretreatment of VEGF receptor inhibitor blocked S1P-induced mES cells proliferation, but not VEGF neutralizing antibody or VEGF siRNA, which suggests that the S1P-induced proliferation was mediated by direct activation of Flk-1 as well as VEGF production. In addition, S1P increased phosphorylation of ERK and JNK in a time-dependent manner, which were blocked by pretreatment of VEGF inhibitor but VEGF neutralizing antibody and VEGF siRNA did not affect. Further, inhibition of ERK and JNK with pharmacological inhibitor blocked S1P-induced mES cells proliferation. In conclusion, S1P-elicited transactivation of Flk-1 mediated by S1P1/3-dependent β-arrestin/c-Src stimulated mES cells proliferation through activation of ERK and JNK in mES cells.

Poster Board Number: F-3034

MOLECULAR INFORMATION PROCESSING DURING A STEM CELL FATE TRANSITION

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Decisions in stem cell fate are critical in development and can be pictorially described in the form of the Waddington Landscape. Underlying the choices present in that landscape are complex multidimensional processes governing cellular phenotype transitions. Here we have studied the temporal flow of information inherent in a Waddington Landscape via analysis of histone code, DNA methylome, messenger RNAs, microRNAs, and proteins in response to induce differentiation by the downregulation of Esrrb. We demonstrate the existence of complex integrated patterns and feed forward regulatory loops regulated by Esrrb across the different regulatory layers. Thus, complex informational flow through different molecular regulatory layers defines one path through the Waddington Landscape whilst integration and comparison of our results with previous Nanog studies revealed a differential equally effective network module. These analyses of early fate mapping give quantitative modelling of Wadsworth landscaping to enable systematic understanding of cellular development from a novel perspective.

Poster Board Number: F-3035

THE ROLE OF E-CADHERIN IN MOUSE EMBRYONIC STEM CELLS

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E-cadherin is a cell adhesion molecule with well-established roles during tumorigenesis, tissue formation and embryonic stem

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(ES) cell pluripotency. Its location at the cell surface has made E-cadherin an attractive target for the manipulation of ES cell culture however a more detailed understanding of the specific downstream processes involved in E-cadherin function is required to fully exploit its therapeutic and commercial potential. We have recently performed microarray analysis to compare E-cadherin null (Ecad-/-) mES cells and wild type (wt) D3 mES cells, identifying >2000 changes in transcripts associated with a wide range of biological processes, including primary metabolic processes, cell differentiation and apoptosis. In order to distinguish between genes regulated by E-cadherin and those associated with loss of epithelial integrity a second microarray was performed comparing E-cadherin Negative Proliferating Stem (ENPS) cells (generated by LIF withdrawal) to their wt counterpart (ENPS reversed [ENPS^R] mES cells). Transcript analysis of two independently derived ENPS cell lines also revealed >2000 significant alterations compared to ENPS^R mES cells. In this study, we compare significant gene expression changes between all E-cadherin negative cell lines, identifying a subset of up- and down-regulated genes common to all three cell lines and revealing that ~200 upregulated and ~400 downregulated transcripts are shared between transcriptomes of Ecad-/-, ENPS1 and ENPS2 mES cells compared to their wt counterparts. Many of these transcripts represent genes involved in epigenetic regulation, transcription factor activity and apoptosis along with being associated with a range of signalling pathways associated with cell growth and proliferation. Further analysis of gene expression changes related to the loss of E-cadherin will provide an insight into the specific processes and pathways that E-cadherin regulates, thus aiding our understanding of its role in ES cell behaviour.

Poster Board Number: F-3036

THREONINE METABOLISM REGULATES HISTONE METHYLATION IN PLURIPOTENT EMBRYONIC STEM CELLS

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Threonine is uniquely critical for the self-renewal and pluripotency of mouse embryonic stem cells (mESCs) but by an unknown mechanism. Here we demonstrate that threonine (Thr) and methionine (Met) metabolism are coupled in pluripotent stem cells, resulting in regulation of S-adenosyl-methionine (SAM) levels and histone methylation. Isotope labeling of mESCs revealed that Thr provides a significant fraction of both the methyl groups and the reducing power needed for SAM synthesis. Depleting Thr from the culture medium or knocking down expression of threonine dehydrogenase (Tdh) in mESCs resulted in decreased SAM levels, reduced tri-methylation of Lysine 4 of histone H3 (H3K4me3), slowed growth, and increased differentiation. Ectopic expression of Tdh in human fibroblasts, which lack a functional Tdh gene, increased SAM and H3K4me3 levels and enhanced reprogramming to the pluripotent state. These findings demonstrate that metabolic regulation of cellular SAM levels play a critical role in determining the extent of H3K4 trimethylation, and illustrate a novel mechanism by which modulation of a critical cellular metabolite influences the pluripotent state.

Poster Board Number: F-3037

AURKA-MEDIATED P53 PHOSPHORYLATION REGULATES EMBRYONIC STEM CELL PLURIPOTENCY AND SOMATIC CELL REPROGRAMMING

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Embryonic stem cells (ESCs) are derived from the inner cell mass. ESCs can be propagated indefinitely and are able to differentiate into all cell lineages of an organism. Many signals must be integrated to maintain self-renewal and pluripotency in ESCs and to enable induced pluripotent stem cell (iPSC) reprogramming. However, the exact molecular mechanisms by which cell fate changes are controlled are still elusive. To unravel the essential internal and external signals required for sustaining the ESC state, we conducted a short hairpin (sh) RNA screen of 104 ESC-associated phosphoregulators and identified aurora kinase A (Aurka). Depletion of Aurka resulted in compromised self-renewal and consequent differentiation. By integrating global gene expression and computational analyses, we discovered that loss of Aurka leads to an up-regulation of p53 signaling which triggers ESC differentiation. Specifically, Aurka regulates pluripotency through inhibition of p53-directed ectodermal and mesodermal gene expression. Aurka phosphorylates p53 at two specific sites, S212 and S312 in mouse ESCs. We report here that phosphorylation of S212, but not S312 plays a major role in impairing p53-induced ESC differentiation, as well as hampering p53-mediated suppression of iPSC reprogramming. In conclusion, our findings are highly significant because they reveal that a mitotic kinase, Aurka, controls a non-mitotically regulated substrate, p53, in ESCs. This is the first report demonstrating that a specific phosphorylation event regulates ESC self-renewal, differentiation, and somatic cell reprogramming.

Poster Board Number: F-3038

GENOMIC INTEGRITY SAFEGUARDS PLURIPOTENCY IN EMBRYONIC STEM CELLS

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Embryonic stem cells (ESCs) hold great promise for biomedicine as a major source for generating differentiated cells for transplantation-based therapies as well as for developing new avenues to study the etiology of diseases. It is both urgent and essential to have a comprehensive understanding of the molecular mechanisms controlling ESC self-renewal and the decisions governing differentiation into a wide range of mature cells for ESCs to fulfill their promise in regenerative therapy. Recent studies extensively or at least primarily focus on the functions of core transcription factors and epigenetic modifications involved in maintaining the undifferentiated stem cell state, ESC self-renewal and pluripotency; however, a global picture of cellular mechanisms controlling ESC identity remains incomplete. In addition, there is little information concerning how ESC "specific" regulatory molecules interface with more "generic" regulators of cellular physiology. To explore the complexities of ESC self-renewal machinery, we first applied a short hairpin (sh) RNA functional genomics screening strategy to identify the essential protein kinases and phosphatases (PKases and PPases), the central regulators of signal transduction, that are required

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for maintaining ESC self-renewal and pluripotency. We found that 5 phosphoregulators have roles in maintaining ESC identity. None of these have previously been implicated in such regulatory functions. In order to elucidate the underlying mechanisms by which these 5 phosphoregulators control ESC self-renewal, we applied systematic approaches to integrate the changes in genome-wide gene expression profiles upon depletion of these 5 phosphoregulators using a variety of computational approaches. These analyses demonstrate the importance of maintaining genome stability in ESC self-renewal. Indeed, depletion of these 5 phosphoregulators led to the accumulation of DNA damage and genomic abnormalities. We further examined the effects on ESC self-renewal after depletion of numerous genome maintenance and stability-associated families, including molecules involved in DNA replication and checkpoints, mRNA processing, Fanconi anemia, and Charcot-Marie-Tooth disease associated gene-products. We show different degrees of compromised ESC self-renewal and pluripotency upon depletion of these molecules. These findings suggest that the maintenance of genome integrity is essential for ESC self-renewal and pluripotency. Furthermore, the decision by ESCs to transition from pluripotency to differentiation may be a novel mechanism to effectively remove compromised stem cells from a position of dramatic organismal damage potential. In summary, our studies reinforce the need to develop safe and effective methods for utilizing ESCs for regenerative medicine. Additionally, understanding the basic molecular mechanisms underlying ESC regulation will provide invaluable information to bring induced pluripotent stem cells (iPSCs) towards future clinical applications.

Poster Board Number: F-3039

DEVELOPMENT OF A FEASIBLE VERIFICATION SYSTEM FOR PLURIPOTENT STEM CELLS USING PORCINE PARTHENOGENETIC EMBRYOS

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There are two types of pluripotent stem cells: naïve and primed. Chimera formation ability is an evident indicator of the naïve state of pluripotent stem cells. We aimed to develop an efficient system to verify the cell naivety by aggregation with parthenogenetic embryos. Porcine parthenogenetic embryos derived from in vitro matured oocytes at the 4- to 8-cell or morula stage were utilized as host embryos after enzymatic removal of the zona pellucida. Inner cell mass (ICM) cells isolated from porcine parthenogenetic blastocysts by immunosurgery were labeled with fluorescent carbocyanine dye (DiI) and used as donor cells. A donor ICM was aggregated with 2 host embryos in a micro-well (400 µm diameter, 300 µm deep) and cultured in vitro for 2 to 3 days. Development of the aggregated embryos into blastocysts and incorporation of the donor ICM cells into the resultant blastocysts were evaluated. ICM cells isolated from in vitro-fertilized porcine blastocysts harboring humanized Kusabira-Orange (huKO) gene were also used as donor cells, and the resultant blastocysts were transferred to 4 recipient gilts to collect fetuses on day 18. Aggregated embryos efficiently developed to blastocysts regardless of the stages of host embryos (4-8-cell: 23/26, 88.5%; morula: 23/24, 95.8%), and the efficiency of incorporation of the donor cells into the ICM were also high (17/23, 73.9% and 20/23, 87%). Transfer of 54 blastocysts developed from 4-8 cell embryos to 2 recipients gave rise to 22 (40.7%) fetuses, of which 3 (13.6%) were confirmed to be chimeric by their clear orange fluorescence and immunostaining. Of 25 (34.2%) fetuses obtained after transfer of 73 blastocysts derived from morulae to 2 recipients, 6 (24.0%) were chimeric. Contribution of the donor cells in the tissues of the chimeric fetuses measured by image analysis software (imageJ) ranged between 16.1% and 65.2%. These data indicate that in vitro and *in vivo* analysis after aggregation with porcine parthenogenetic embryos is a feasible method for verification of pluripotent cells such as ES and iPS cell.

Poster Board Number: F-3040

ERK MAP KINASE SIGNALING TARGETS NANOG MRNA FOR DEGRADATION

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FGF/Erk MAP Kinase signaling plays a key role in regulating Mouse Embryonic Stem Cell pluripotency. However, the mechanistic connection between signaling pathway activity and pluripotency associated transcription factors remains unclear. Here we show that FGF signaling regulates Nanog mRNA levels via Zfp36 family RNA binding proteins (TTP, Brf1 and Brf2). FGF/Erk MAP kinase signaling activates the expression of Zfp36 proteins, which bind to AU-rich response elements in the Nanog 3'UTR, rapidly targeting it for degradation. Zfp36 proteins also affect cell fate regulators more broadly, downregulating the expression of other core pluripotency genes (such as Oct4, Klf4 and Rex1), and specifically upregulating primitive endoderm markers (Gata6 and Dab2). These results provide a post-transcriptional pathway for the control of pluripotency and differentiation in response to FGF signals.

Poster Board Number: F-3041

EVOLUTIONARY BACKGROUND OF MOLECULAR MECHANISMS OF PLURIPOTENCY: FGF/ERK-INDEPENDENT PROLIFERATION TO MAINTAIN PLURIPOTENCY IN EARLY EMBRYOS

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Investigating the evolution of pluripotency will provide insights into the origin of multicellular development and deeper knowledge for medical applications of human pluripotent stem cells. It would also give hints for how to approach problems such as the attempt to obtain naïve state human ESCs. Recent findings have began to clarify the roles and the relationships of one positive regulatory path and two negative regulatory paths of the pluripotent state: (1) a transcription network activated by LIF/STAT3 signaling to support propagation in the undifferentiated state, (2) FGF/ERK signaling and (3) pathways related to GSK3/β-catenin to initiate differentiation. These molecular mechanisms of pluripotency have been studied mainly in mammalian systems to date. Thus, the factors and molecular pathways underlying pluripotency in other vertebrates, e.g., birds or reptiles, are not yet well understood. Since they can reprogram somatic cells to iPSCs, pluripotency transcription factors are thought to be the most important part of the molecular mechanism of mammalian pluripotency. However, the binding sites of Oct3/4 and Nanog are not highly conserved between mammalian genomes and the chicken genome. Furthermore, an orthologue of *Oct3/4* has not been found in the chicken genome, but was found in the genome of an anole lizard. These diversifications of the critical pluripotency transcription factors raise interest-

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ing questions about the transcription network in these species, and at the same time, obscure the essential part of the mechanism. To study the evolutionary background of pluripotency, we observed the effects of extracellular effectors on primary culture cells from avian and reptile embryos. First, we focused on the negative regulations of pluripotency. When treated with a MEK inhibitor and a GSK3 inhibitor (2i condition), chicken early embryos formed domed colonies (DCs), which were morphologically indistinguishable from mouse and rat ESCs. However, we could not obtain any DCs when we seeded cells from more-developed embryos in the 2i condition, indicating that there is a clear boundary of DC-forming ability at around primitive streak formation. Quail embryos taken from eggs before and after oviposition also formed DCs in the 2i condition. Gecko blastoderms also formed DCs in the 2i condition, but gastrulas did not. Chicken embryonic cells cultured in the 2i condition showed high expression levels of germ-line-specific genes, probably reflecting their high developmental potential. Interestingly, chicken homologues of the mammalian pluripotency transcription factors were not highly expressed in the 2i condition.

Poster Board Number: F-3042

DAX1 ASSOCIATES WITH ESRRB AND FUNCTIONS AS A REPRESSOR IN EMBRYONIC STEM CELLS

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Pluripotency and self-renewal capacity are major characteristics of embryonic stem (ES) cells, which are regulated by Oct3/4-centered protein-interaction network. Nuclear hormone receptor Dax1 is one of the essential factors in the network. In this study, we identified an orphan nuclear receptor Esrrb as a Dax1-interacting protein. Expression of Esrrb was specific for undifferentiated ES cells and regulated by Oct3/4. Interaction between Dax1 and Esrrb was mediated through the 3rd LXXLL motif of Dax1 and the activation- and ligand-binding domains of Esrrb. Pull-down assay revealed that Dax1 selectively binds to either Esrrb or Oct3/4. Luciferase and biotin-labeled DNA pull-down assays clarified that Esrrb enhances promoter activity of the *Dax1* gene and binds to an ERRE1 site of the promoter. Moreover, transcriptional activity of Esrrb was repressed by Dax1. Expression of Dax1 was down-regulated followed by Oct3/4 repression; however, overexpression of Esrrb maintained expression of Dax1 even in the absence of Oct3/4. Knockdown of Esrrb induced repression of Dax1 expression. These data together with previous findings suggest that Dax1, whose expression is regulated by Oct3/4 and Esrrb, functions as a negative regulator of Esrrb and Oct3/4, and these molecules form a regulatory loop for controlling pluripotency and self-renewal capacity of ES cells.

Poster Board Number: F-3044

ESCC MIRNAS SUPPRESS THE RESTRICTION POINT IN EMBRYONIC STEM CELLS

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The restriction (R) point is a control point in the G1 phase of the cell cycle, before which a cell relies on mitogenic factors for cell cycle progression. Embryonic stem (ES) cells like cancer cells lack the R point and self-renew independently of mitogenic factors, despite that ES cells are non-transformed cells. The mechanisms underlying the suppression of the R point in ES cells is not well understood. To identify possible roles of microRNAs (miRNAs), we analyzed the cell cycle progression in Dgcr8 and Dicer knockout ES cells that lack canonical miRNAs, under serum starvation, contact inhibition, and DNA damage conditions. MiRNA-deficient cells were significantly accumulated in the G1 phase under serum starvation or contact inhibition treatments, but not under DNA damage conditions; although significant increase of apoptosis was observed at all conditions. Epidermal growth factor partially suppressed the G1 accumulation phenotype. ESCC miRNAs that promote the G1-S transition and proliferation of ES cells under normal growth conditions also prevent the G1 arrest in miRNA-deficient cells under serum starvation and contact inhibition conditions. Interestingly, these miRNAs are significantly upregulated in serum starvation conditions. Triple knockout of Rb1, Rbl1 and Rbl2, targets of ESCC miRNAs, completely blocked the G1 arrest, indicating that ESCC miRNAs suppress the R point by targeting the Rb pathway. Our data support that ESCC miRNAs play an important role in the suppression of the R point and facilitate the mitogenic factor-independent growth of ES cells. The similar mechanism is likely hijacked by cancer cells to bypass the R point control.

Embryonic Stem Cell Clinical Application

Poster Board Number: F-3045

RETINAL PIGMENT EPITHELIUM DERIVED FACTOR INHIBIT GROWTH OF HUMAN IPS CELLS AND MOUSE ES CELLS.

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One of the major safety concerns in pluripotent stem cell-derived cell therapy is a residual of undifferentiated cells, which resulted in causing teratoma or tumor after transplantation. However a safety issue of this sort shall be addressed case by case depending on the terminally differentiated cell types. Here we show that retinal pigment epithelium (RPE) derived factor dramatically inhibited the iPSCs growth. The iPSCs in co-culture with RPE caused morphological changed, drastically reduced the expression levels of Oct3/4 and Nanog and eventually induced apoptotic cell death. To address this event *in vivo*, several doses of iPSCs were mixed with RPE in matrigel and inject into the immunosuppressant NOG mice subcutaneously. Injection of 1x10^3 iPSCs alone was enough to generate subcutaneous tumor, but the formation of tumor was significantly reduced if the same dose of iPSCs were injected with 1x10^6 cells of primary or iPSC-derived RPE. These results indicate a safety issue of cell transplantation of iPSC-derived RPE can be discussed separately from other iPSC-derived cell transplantation therapies. Pigment epithelium derived factor (PEDF) was detected in the conditioned medium of both primary and iPSC-derived RPE. Some reports suggest that PEDF causes a profound inhibition of tumor growth in animal cancer models. Addition of recombinant PEDF inhibited the growth of mouse ES cells, whereas neutralizing PEDF antibody promoted the growth of these cells, confirming that the growth-inhibitory effect was PEDF specific. However iPSCs with recombinant PEDF in culture medium maintained an undifferentiated state and caused no significant change of growth. Now we investigate whether PEDF is an indirect effect of the reduction of the iPSCs growth through a different pathway in the mouse ES cells.

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Poster Board Number: F-3046

EPIGENETIC STABILITY, ADAPTABILITY, AND REVERSIBILITY IN AN UNDIFFERENTIATED HUMAN EMBRYONIC CELL LINE

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The stability of human embryonic stem cells (hESC) is of critical importance for both experimental and clinical applications. In this work, we sought to assess adaptability and reversibility of DNA methylation in hESCs undergoing adaptive responses to shifting culture environments. In consideration of the wide-ranging therapeutic value inherent to hESCs, we adapted cells to defined or defined and xeno-free alternatives to feeder-based cultures and monitored accompanying changes in DNA methylation and gene transcription. Our results indicate changes to DNA methylation to reflect culture environment, to influence gene expression, and although most DNA methylation changes are culture specific and reversible, some are retained as a "memory" of culture history. For these culture-induced methylation changes, we note a novel correlation: hypomethylation of regions 500-2000 bp upstream of promoters correlates with decreased expression, opposite to that commonly seen at proximal promoters. Lastly, the identification of several differential methylated regions at key G-protein signaling and developmental genes imply that even a single DNA methylation event can have profound influence on transcription and differentiation propensity.

Poster Board Number: F-3047

PARALLEL ASSESSMENT OF A GLOBIN LENTIVIRAL VECTOR AFTER TRANSDUCTION OF IPS AND SOMATIC HEMATOPOIETIC STEM CELLS FROM THE SAME TRANSPLANTED HUMAN B-THALASSEMIA PATIENT

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Patients with -thalassemia major require lifelong transfusions regardless of the type of causative mutations (e.g., β0, βE/β0). The only available curative therapy is allogeneic hematopoietic transplantation, although most patients do not have an HLA-matched, geno-identical donor, and those who do still risk engraftment failure and graft-versus-host disease. Hence, gene therapy by *ex vivo* transfer of a functional globin gene into the patient's own somatic hematopoietic stem cells (HSCs) is an attractive novel therapeutic modality. Lentiviral vectors have proven especially suited for this application. Hence, the first Phase I/II human clinical trial was initiated by our group, and the first treated βE/β0-thalassemia patient previously suffering from a severe clinical form has become transfusion independent for the past 3.5 years (Nature, 2010). However, potential oncogenic genotoxicity in some patients remains a concern due to the intrinsic quasi-random nature of lentiviral integration. This is why globin lentiviral transfer to human induced pluripotent stem cells (iPSCs) is an attractive alternative, because one can isolate sub-clones where the vector has integrated in relatively "safe" areas of the human genome or directly perform genetic correction by homologous recombination/repair. However, a multitude of unknowns and hurdles remain before transplantation of iPSCs or their derivatives becomes a viable approach, and comparing HSCs derived from human iPSCs with their natural isogenic somatic counterparts had not been performed in the context of therapeutic gene delivery. Here, mesenchymal stem cells from a βE/β0-thalassaemia patient who had been treated with gene therapy were reprogrammed into iPSCs by transduction of retroviruses carrying 4 transcription factors: Oct-4, Sox-2, Klf-4, and c-Myc. iPSC subclones were then transduced with the same batch of βA(T87Q)-globin lentivector used in the clinical trial. We first examined the theoretical oncogenic risk of lentiviral globin vector integration after DNA pyrosequencing by determining the positions of integration sites relative to known genes, oncogenes, tumor suppressor genes, miRNA genes or ultraconserved regions. Approximatey 15% of integration sites were in relative "safe" areas. Because it is known that persistently high expression of fetal (γ) hemoglobin would be sufficient to treat thalassemia and sickle cell anemia, we quantified embryonic, fetal and adult globin gene expression in Thal-iPS-derived erythroid cells in vitro and in vivo after transplantation into immunodeficient (NSG) mice to determine whether γ-globin expression is irreversibly "blocked" after passage through an iPSC state. Multi-lineage engraftment in immunocompromised mice, and embryonic-fetal together with partial fetal-adult globin class switching were observed. Surprisingly, common integration sites were identified across iPSC lines and cells retrieved from isogenic and non-isogenic gene therapy patients with β-thalassemia and adrenoleukodystrophy, respectively. Common integration sites observed in the absence of overt tumorigenesis thus result from non-random lentiviral integration rather than oncogenic in vivo selection. These findings bring the use of human iPSCs closer to practicality and further clarify both mechanics and interpretation of genome-wide lentivector integration.

Poster Board Number: F-3048

MICROENCAPSULATION OF HUMAN PANCREATIC PROGENITORS AS A PREVENTITIVE STRATEGY FOR FORMATION OF TERATOMAS FROM RESIDUAL PLUIRPOTENT CELLS

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Microencapsulation of human pancreatic progenitor (hPP) cells in a biomaterial prior to their transplantation is a strategy being used to provide a treatment for diabetes. Protecting the cells with a molecular weight selective material may reduce the need for concurrent immunosuppressive therapy. Previously we have shown that encapsulation prevents the formation of teratomas when pluripotent stem cells are transplanted into immunodeficient mice (Transplantation 2006; 82: 1175). In that study, we maintained encapsulated human embryonic stem cells (hESC) for 4 weeks. In the current study, hPP derived from hESC were transplanted into NOD/SCID mice and monitored for up to 4 months. Cells were either encapsulated in alginate and injected into peritoneal cavity or mixed with a blood clot and placed under the kidney capsule. We were interested to determine if encapsulation prevented teratoma formation from the unpurified PP that had differentiated from hESC. Pluripotent hESC were differentiated into PP using a modification of the published technique of Kroon et al (Nat Biotech 2008; 26: 443). On the final day of differentiation, >70% of cells stained positively for PDX-1 and >50% for SOX9. qPCR analysis showed an up-regulation of the genes for endocrine and precursor pancreas, PDX-1, SOX9, HNF6, NKX6.1, NGN3 and NKX2.2 during the culture period, peaking at the end of the differentiation process. Markers of pluripotent stem cells, Oct 4 and Nanog, were down-regulated during differentiation, the level of expression in PP being ≤ 1% of those in pluripotent cells. PP were either encapsulated in barium alginate (2.2%) and infused into the peritoneal cavity of 16 NOD/

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SCID mice (n=16), or mixed in a blood clot and grafted beneath the renal capsule (n=16). Mice were monitored regularly and euthanized at 8, 12 and 16 weeks post transplantation, or when the mice lost >20% of their body weight. At post-mortem, the encapsulated cells were retrieved from the peritoneal cavity for analysis and the kidney grafts examined for teratoma formation using routine histology. Teratomas, which express ecto-, meso- and endoderm, were detected in 3 of the 16 mice that received renal grafts at 12-13 weeks post transplantation. No teratomas formed in any mice that received encapsulated PP. Not unexpectedly for immunodeficient animals, thymomas developed in both the renal graft group (3/16) and encapsulated cell group (1/16). A control group of mice (n=4) was established in which hESC were grafted beneath the renal capsule. Teratomas formed in each of these mice (4/4), necessitating euthanasia at 4 - 7 weeks post-transplantation. Data showed that 1) residual pluripotent stem cells in PP population could cause teratoma formation when grafted under the kidney capsule (at a rate of at least 19%) and 2) encapsulating PP prior to transplantation prevented teratoma formation (0% rate).

Poster Board Number: F-3049

MANUFACTURING AND BANKING OF CLINICAL-GRADE HUMAN EMBRYONIC STEM CELL LINES IN A GMP FACILITY

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Five human embryonic stem cell (hESC) lines established in the Institute of Frontier Medical Sciences, Kyoto University, have been supplied to researchers in Japan complying with national regulations. Manufacturing clinical-grade hESC lines and their banking requires various factors for the safety of recipients, such as development of xeno-free culture conditions with maintenance of cell characteristics, viral screening, documentation of cell processing procedures, maintenance of GMP facility, etc. For production of clinical-grade hESC lines, a cell processing center was built in 2006 in the Institute comprising a clean room for establishment and expansion of hESC lines, a room for an Isolator (Cell Processing Isolator, Sanyo) in which cells can be processed and cultured in a completely closed system, a cell-storage room, a computer-controlled observation room, a supply room, and a laboratory operating with GMP compliance. Validation of cell processing facility has been performed periodically to certify that the environment of cell production meets the requirements for clinical-grade hESC line production. Cleaning up procedures of existing hESC lines, i.e., continuous culture with xeno-free conditions (free from use of feeder-cells and animal serum) and defined culture medium have been studied to avoid risk of contamination with animal derived virus. Chromosomal and genetic stability, maintenance of undifferentiated status, pluripotency of the cells during cleaning up procedures have been tested. Human and animal viral tests have been performed to avoid the risk of contamination. The procedures employed follow ISCBI (International Stem Cell Banking Initiative) guidelines we participated in establishment and were published in: "Consensus guidance for banking and supply of human embryonic stem cell lines for research purposes" (Stem Cell Rev and Rep 5, 301, 2009). It is concluded that most of the items described in the guidance document are applicable for production of clinical-grade hESC lines.

Poster Board Number: F-3050

NURR1 AND CA-RAF TRANSDUCED NPCS YIELD GRAFTS ENRICHED WITH DOPAMINERGIC NEURONS AFTER TRANSPLANTATION INTO RAT BRAINS.

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Nurr1 is an orphan nuclear receptor exressed in dopamine neurons of developing and adult midbrains. Engineering of Nurr1 in NPCs generates DA neurons with promoted cell survival and resistant to toxic stimuli. Intrastriatal transplantation of Nurr1-transduced NPCs generates DA cell survived and integrated into host brain. However, the enrichment of DA cells in the grafts generated by Nurr1-transduced NPCs was only transient but not sustained for long period of post-transplantation. We show herein that activation of Raf-ERK signaling in these donor cell by co-expression of constitutive active(ca) Raf contribute to a faithful generation of large grafts fully enriched with DA cells after transplantation. The ca-Raf co-expression effect was achieved by multiple mechanisms such as Nurr1 mRNA expression by CREB activation, and cell survival and proliferation.

Embryonic Stem Cell Differentiation

Poster Board Number: F-3051

MESODERM COMPARTMENTALIZATION FOR DISTINCT ENDOTHELIAL CONTRIBUTION TO EMBRYONIC VASCULATURE

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Endothelial cells are the fundamental cell type generated from mesoderm to support embryonic development. However, how primitive mesoderm is organized to build specific part of the embryonic vasculature at later developmental stage has not been well defined. Using Etv2/ER71 as a clue, a master regulator to create endothelial cells from mesoderm, we tried to probe the contribution of specific mesoderm subset to future embryonic vessels during development. Since Etv2 is transiently expressed and presumably required only in the restricted timeframe of endothelial commitment from primitive mesoderm, ablating Etv2 in early specific mesoderm subset will provide an ideal way to evaluate the contribution of primitive mesoderm to vascular system. We found that mesoderm subset of specific region contributes to the corresponding part of the vasculature without little or no compensatory migration from other areas. For example, Etv2 ablation in extraembryonic and anterior mesoderm led to the loss of endothelial cells in yolk sac, rostral part of the embryo, and endoardium, saving caudal part of the embryo relatively intact. In particular, we sought to define the primitive mesoderm that contributes to hemogenic endothelial cells. Defining the origin of specific endothelial cells in early embryos will help to get fundamental information to guide endothelial cell generation from ES cells.

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Poster Board Number: F-3052

DERIVATION OF HEMATOPOIETIC CELLS FROM PDGFR ALPHA POSITIVE MESODERM SUBSET

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Embryonic origin of hematopoietic stem cells is yet to be clearly defined. Recently, it has been reported that all hematopoietic cells are derived from Flk-1+ cells. Our previous work, however, suggested that PDGF receptor alpha+ cells, that comprise the distinct mesoderm from Flk-1+ population, may also generate hematopoietic cells in in vitro differentiated ES cells. To pursue this possibility *in vivo*, we have created mice that express Tamoxifen inducible Mer-Cre-Mer under control of the PDGF receptor alpha locus. IWe have found that PDGF receptor alpha+ cells pulse labeled in early mesoderm contribute to B lymphocytes and stem cell populations in fetal liver. This result demonstrated that PDGF receptor alpha+ mesoderm can give rise to hematopoietic cells that contribute to part of the definitive hematopoiesis. We also found that some endothelial cells were also generated from PDGF receptor alpha+ cells. These results indicate the plasticity of early mesoderm and raise the possibility that hematopoietic and endothelial cells may be induced from PDGF receptor alpha+ cells such as mesenchymal stem cells.

Poster Board Number: F-3053

EFFECT OF EARLY ENDODERM INDUCTION IN LATE PANCREATIC COMMITMENT DURING DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS

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Pancreatic differentiation of mouse and human embryonic stem cells has received considerable attention over the last decade. While there has been some success in deriving insulin positive cells from embryonic stem cells, typically the differentiated cells are limited in yield and functionality. Most of the successful differentiation protocols involve a step-wise directed differentiation strategy starting with definitive endoderm (DE) induction; followed by pancreatic progenitor induction and finally islet maturation. While extensive studies have established multiple pathways of endoderm induction, the effect of these pathways on late stage pancreatic maturation remains elusive. The aim of this study is to develop a thorough understanding of the effect of pathway of early endoderm induction on late stage pancreatic maturation. The human embryonic stem cells were differentiated to mature insulin producing cells following a directed differentiation approach. The first stage of definitive endoderm was induced following four alternative pathways, based on literature reports. Namely, we used Activin A in combination with one of the following molecules and growth factors: Fgf2, Bmp4, Wnt3a or PI3K Inhibitor. DE derivatives obtained from these four pathways were subjected to same maturation protocol: pancreatic progenitor induction using sonic hedgehog inhibition and retinoid signaling; islet maturation through notch inhibition. The differentiation potential of each of the endoderm derivatives were compared at different stages of differentiation. Our results clearly indicate that late stage pancreatic maturation is distinctly governed by the initial pathway of endoderm commitment. Also it reveals that analysis of endoderm is not a good indicator of subsequent pancreatic maturation. From the results obtained from 6 independent studies, we found that DE induction by treatment with PI3KI resulted in the strongest upregulation of DE markers Sox17, Foxa2 and Cxcr4; however upon maturation it resulted in a moderate 800-fold upregulation of insulin. Conversely Wnt3a and Fgf2 resulted in moderate expression of DE markers, but upon maturation showed between 10000 and 20000 fold upregulation of insulin. Consistent with PCR analysis, Wnt3a led to highest yield of C-peptide expressing cells with around 25% positive cells. Interestingly, while the Bmp4 induced DE cells resulted in only about 2-fold increase in insulin expression, they showed the highest upregulation of glucagon: around 4000 times higher than undifferentiated cells, leading to the conclusion that inducing DE with Bmp4 leads to more alpha cells than beta cells. We conclude that using Activin A in combination with Wnt3a at the definitive endoderm stage is the most effective strategy for beta cell maturation, while BMP4 induced endoderm results in higher potential for alpha cell maturation. Furthermore, we performed a detailed principal component and correlation analysis, which reveals a low correlation between endoderm specification and pancreatic maturation with the tested markers. Known pancreatic progenitor markers, however, were found to correlate strongly with islet maturation.

Poster Board Number: F-3054

ENDOTHELIAL CELL MEDIATED MATURATION OF HUMAN EMBRYONIC STEM CELL (HESC) DERIVED PANCREATIC PROGENITOR INTO INSULIN EXPRESSING CELLS

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Diabetes and its complications affect 25.8 million people in the US and is a growing healthcare problem worldwide. While transplantation of pancreatic islets offer potential therapy, it is restricted by scarcity of donor islets. Effective use of hESC could overcome this limitation, however current approaches to differentiating hESCs to mature islets have been restricted in yield and functionality of the differentiated phenotype. In our work we propose to mimic in-vivo development during maturation of hESC derived pancreatic progenitor cells. Over past years developmental studies have established the critical involvement of endothelial cells in maturation and functionality of pancreatic islets, which is not surprising given the islet's dense vasculature. We have developed a multi-stage protocol for directed differentiation of hESCs involving definitive endoderm induction, followed by pancreatic progenitor specification and final maturation into insulin producing cells. For the last step, we propose to co-culture the hESC-derived pancreatic progenitor cells with endothelial cells in accordance with developmental studies. We used two different cell types in our studies: Rat Heart Microvascular Endothelial Cells (RHMVEC) and Human Umbilical Vein Endothelial Cells (HUVEC). As a control experiment we co-cultured the pancreatic progenitor cells with NIH3T3 fibroblast to verify sensitivity to cell types. All direct contact co-cultures were FACS sorted and analyzed for gene and protein expression levels. Two different non-contact co-culture configurations were also investigated: transwell co-culture and endothelial cell conditioned media. Results were evaluated with respect to conventional maturation using notch inhibition by DAPT. We evaluated each stage of differentiation using qRT-PCR, immunostaining and flow cytometry. Our protocol resulted in 89% Foxa2 posivite endoderm and high, uniform nuclear staining for Pdx-1 at pancreatic progenitor stage. Maturation with DAPT resulted in 50 fold upregulation of insulin with 8% commitment, while parallel RHMVEC co-culture resulted in 400 (+- 100) fold upregulation of insulin with 34% commitment. HUVEC co-culture resulted in an even higher insulin expression, 20

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folds higher than RHMVEC. Control cultures with fibroblast did not show appreciable insulin upregulation, indicating the effect to be specific to endothelial cells. Alternative co-culture configurations also show promising results, with trnaswell dominating over conditioned media, but still lower than direct contact configuration. Comparison of C-peptide secretion shows 3 times higher levels compared to DAPT treatment. Our results establish an alternate strategy for hESC maturation into islet cell types. Current investigation is underway to elucidate the mechanism of endothelial cell mediated maturation.

Poster Board Number: F-3055

MECHANICAL FORCE AND SCLERAXIS SYNERGISTICALLY PROMOTE THE COMMITMENT OF HUMAN EMBRYONIC STEM CELLS TO TENOCYTES FOR FUNCTIONAL TENDON REGENERATION

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Derivation of tenocytes from human embryonic stem cells (hESCs) represents a promising approach to functional repair of tendon injuries. However, strategies that specifically drive differentiation of hESCs into tenocyte for tendon regeneration have yet to be defined. Here, we report that overexpression of the tendon-specific transcription factor scleraxis (SCX) combined with mechanical stress synergistically promoted commitment of hESC-derived mesenchymal stem cells (hESC-MSCs) to tenocyte. While mechanical stimulus alone increased tenocyte gene expression, it concomitantly activated osteogenesis mediated by BMP signaling pathway. However, overexpression of SCX antagonized BMP signaling cascade and, together with mechanical stimulus, synergistically augmented teno-lineage differentiation by selectively inhibiting osteo-lineage differentiation program in vitro and *in vivo*. Our study not only demonstrated a novel strategy of combing genetic and physical methods for directing hESC differentiation to tenocyte for functional tendon regeneration, but also offers insights into developing novel therapeutic approach to prevent pathological ossification of injured tendons for regeneration.

Poster Board Number: F-3056

TRANSPLANTATION OF HUMAN EMBRYONIC STEM CELL-DERIVED PSA-NCAM$^+$ NEURAL PRECURSOR CELLS PROMOTES FUNCTIONAL RECOVERY AFTER CEREBRAL ISCHEMIA IN RATS

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While stroke is a leading disease of death and disability, caused by obstruction or rupture of cerebral vascular vessels, only a few treatment options exist despite intensive research. Thus, a new treatment strategy capable of restoring stroke damage needs to be developed. Here, we have investigated the potential for human embryonic stem cells(hESC) derived PSA-NCAM$^+$ neural precursor cell (NPC) transplantation to repair the ischemic brain. We were able to isolate an expandable and homogenous population of NPCs from hESCs that were fully neuralized and uniformly expressed molecular features of NSCs. Rats were subjected to middle cerebral artery occlusion (MCAo) surgery and allowed to recover for 48hrs before intrastriatal infusion of ~1 x 10^6 PSA-NCAM$^+$ NPCs. Behavioral tests, measurements of brain infarction, and immunohistochemical staining were conducted to quantify neurologic recovery. To increase the objectivity of behavioral test, we adapted quantifiable test results as modified neurological severity score (mNSS). We found that the infarct area as well as mNSS were decreased in PSA-NCAM$^+$ NPC transplanted MCAo brains. The motor and reflex performance of MCAo rats were improved from the 3 days after cell infusion upto 1month. Our results suggest that striatal transplantation of ESC-derived PSA-NCAM$^+$ NPCs appears to restore ischemic brains with MCAo via cell replacement as well as inhibition of inflammation. Thus, hESC-derived NPCs may offer a renewable and homogenous source of neural cells that will be valuable for basic and translational researches. *This study was supported by grant SC-1110 , SC-2130 and MEST(2010-0020353) from the Stem Cell Research Center of the 21st Century Frontier Research Program, the Ministry of Education, Science, and Technology of the Republic of Korea*

Poster Board Number: F-3057

THE INDUCTION OF MALE GERM-LINE CELLS FROM HUMAN EMBRYONIC STEM CELLS AND ITS DIFFERENTIATION INTO HAPLOID GERM CELLS

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Introduction: Human embryonic stem cells (hESCs) have the capacity to differentiate into cells of all three germ layers. Recently, hESCs also can be differentiated into spermatogonial stem cells (SSCs) and male haploid germ cells. However, differentiation efficiency of hESCs into those cell types was very low and it seems to be due to the low early specification rate of hESCs into SSCs. Before the further differentiation into haploid male germ cells, sufficient numbers of SSCs should be prepared. Hence, the purpose of this study was to develop a differentiation condition for enriching of SSCs from hESCs and haploid germ cells. Methods: Embryoid bodies (EBs) were derived from hESCs (CHA-hES4 and H1) in EB medium for 2-3 days of culturing. EBs were cultured for another 2-8days in EB medium with/-out 100ng/ml BMP-4 and 0.1μM RA in order to specify into male germ-lineage cells. Then, those cells were cultured in SSC-proliferation medium (Stem-Pro34 medium contained bFGF, GDNF and LIF) for 7days. For differentiation into haploid germ cell, we isolated SSCs-like cells by MACS using GFR a-1, and the GFR a-1-positive cells were encapsulated with sodium alginate and cultured for 4 days in the differentiation medium (testosterone-based media). After these steps, cultured cells were assessed for SSCs or haploid germ cell-specific markers (integrin α1, integrin β6, VASA, GFR α-1, C-Kit, TH2B and IAP) by using RT-PCR, immunocytochemistry and FACS. Also, microarray was employed to compare gene expression profiles of SSCs from hESCs and SSCs from testis. Results: The specification-rate of male germ-lineage cells (VASA+) was increased in both CHA-hES4 and H1 lines treated with BMP-4 and RA. Co-treated EB cells with BMP-4 and RA have shown a higher rate of VASA+ than those of non- treated groups (24.5%±3.23 and 23.0%±3.49 vs 7.0%±1.29 and 5.6%±1.44, $p<0.05$). The ratio of SSC-specific marker-positive cells in both CHA-hES4 and H1 lines was increased in SSCs-proliferation medium. In the presence of 3 growth factors, population of GFR a-1+ cells were significantly expanded compared to control without the growth factors (21.97% and 23.56% vs 8.42% and 12.66%, $p<0.05$). In the microarray data, SSCs from hESCs showed similar characters of SSC associated genes profiles to SSCs obtained from human testis than undifferentiated hESCs. Of these 140 genes (selected markers from published data), 22 genes were similarly expressed in the three groups (hESCs, SSC from hESCs and SSC from testis). However, 112 genes were only present in SSCs from hESCs and SSCs from

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testis. Finally, increased SSCs were sorted with GFR a-1 and further differentiated by encapsulation in calcium alginate. The expression of spermatocyte or haploid germ cell markers was significantly increased in the cultured cells with differentiation medium compare to control without testosterone and calcium alginate Conclusion: Our culture system for specification, expansion and differentiation was an effective system for production of germ lineage cells from hESCs. Moreover, improvement of this system would provide more information for understanding of germ cell biology and application of hESCs.

Poster Board Number: F-3058

ALGINATE MICROCAPSULE AS A 3D PLATFORM FOR PROPAGATION AND DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS TO DIFFERENT LINEAGES

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Human embryonic stem cells (hESC) are emerging as an attractive alternative source for cell replacement therapy since they can be expanded in culture indefinitely and differentiated to any cell types in the body. Various types of biomaterials have also been used in stem cell cultures to provide a microenvironment mimicking the stem cell niche[1-3]. The latter is important for promoting cell-to-cell interaction, cell proliferation, and differentiation into specific lineages as well as tissue organization by providing a three-dimensional (3D) environment[4] such as encapsulation. The principle of cell encapsulation involves entrapment of living cells within the confines of semi-permeable membranes in 3D cultures[2]. These membranes allow for the exchange of nutrients, oxygen and stimuli across the membranes, whereas antibodies and immune cells from the host that are larger than the capsule pore size are excluded[5]. Here, we present an approach to culture and differentiate hESC DA neurons in a 3D microenvironment using alginate microcapsules. We have modified the culture conditions[2] to enhance the viability of encapsulated hESC. We have previously shown that the addition of p160-Rho-associated coiled-coil kinase (ROCK) inhibitor, Y-27632 and human fetal fibroblast-conditioned serum replacement medium (hFF-CM) to the 3D platform significantly enhanced the viability of encapsulated hESC in which the cells expressed definitive endoderm marker genes[1]. We have now used this 3D platform for the propagation of hESC and efficient differentiation to DA neurons. Protein and gene expression analyses after the final stage of DA neuronal differentiation showed an increased expression of tyrosine hydroxylase (TH), a marker for DA neurons, >100 folds after 2 weeks. We hypothesized that our 3D platform using alginate microcapsules may be useful to study the proliferation and directed differentiation of hESC to various lineages. This 3D system also allows the separation of feeder cells from hESC during the process of differentiation and also has potential for immune-isolation during transplantation in the future.

Poster Board Number: F-3059

MODELING HUMAN MYOFIBRILLAR MYOPATHY WITH INDUCED PLURIPOTENT STEM CELLS

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Myofibrillar myopathies are genetically heterogeneous protein aggregation diseases. The underlying mechanism(s) and effective therapies related to these age-related onset and manifestations remain poorly defined. Mutations in αB-crystallin cause autosomal-dominant inheritable cataract and myofibrillar myopathy. Recently, a female infant was reported with profound muscle stiffness and electromyography characterized by spontaneous electrical activity and pseudomyotonic discharges. Muscle biopsy suggested a myofibrillar myopathy and genetic testing revealed homozygosity for the c.343delT CryAB. To further understand the molecular mechanisms of recessive inherited form of myofibrillar myopathy, the skin fibroblasts from 343delT CryAB patient and healthy control subject were obtained for generation of iPSC. Several clones generated after four-factor reprogramming (OCT4, SOX2, KLF4, and C-MYC) are morphologically indistinguishable from human ESCs. These iPSCs have normal karyotypes, express cell surface markers, express pluripotent genes, and also maintain the developmental potential to differentiate into all three germ layers. We have begun to characterize iPSCs after differentiation into muscle cells and cardiomyocytes. The structure and function of iPS derived-skeletal muscle cells will be reported. In Preliminary studies, the over-expression of 343delT CryAB in H9C2 and rat neonatal cardiomyocyte showed protein aggregation, and also the 343delT CryAB protein levels exist in detergent insoluble fractions compared with WT control. These results suggested that this mutation might cause cardiomyopathy. The protein aggregation studies, cell size measurement, and hypertropic gene expressions will be investigated in enriched iPS derived-cardiomyocyte. In summary, our studies are the first report whether modeling of a protein- aggregation induced myofibrillar disease using human iPSC technology is sufficient to recapitulate certain molecular and biochemical properties found in humans.

Poster Board Number: F-3061

HUMAN NEURAL CREST STEM CELLS DERIVED FROM HESC AND IPSC: INDUCTION,MAINTENANCE AND DIFFERENTIATION INTO FUNCTIONAL SCHWANN CELLS

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The neural crest (NC) is a transient, multipotent, migratory cell population unique to vertebrates that gives rise to a diverse cell lineage. Much of our knowledge on NC development comes from studies of organisms such as chicken and zebrafish as human NC is difficult to obtain because of its transient nature and the limited availability of human fetal cells. Here we examined the process of NC induction from human pluripotent stem cells including embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs).

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We showed that NC cells could be efficiently induced from hESCs by a combination of growth factors in medium conditioned on stromal cells, and that NC stem cells (NCSCs) could be purified by p75 using FACS. FACS isolated NCSCs could be propagated *in vitro* 5 passages and cryopreserved while maintaining the NCSC identity characterized by the expression of a panel of NC markers such as p75, Sox9, Sox10, CD44 and HNK1. *In vitro* expanded NCSCs were able to differentiate into neurons and glia (Schwann cells) of the peripheral nervous system (PNS) as well as mesenchymal derivatives. hESC-derived NCSCs appeared to behave similar to endogenous embryonic NC cells when injected in chicken embryos. Using a defined medium we were able to generate and propagate a nearly pure population of Schwann cells that uniformly expressed GFAP, S100 and p75. Schwann cells generated by our protocol myelinated rat dorsal root ganglia neurons *in vitro*. To our knowledge this is the first report on myelination by hESC- or iPSC-derived Schwann cells.

Poster Board Number: F-3062

A SPECIFIC AMINO ACID METABOLIC STATE OF HUMAN ES/IPS CELLS AND ITS SIGNIFICANCE

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Embryonic stem (ES) cells possess a characteristic high rate of proliferation and a short G1 phase; they have an unlimited ability to replicate as well as the potential to differentiate into most cell types in an organism, including hepatic lineage. The high proliferation rate is considered essential for the maintenance of ES cell identity. Recent studies showed that mouse ES cells are in a high-flux metabolic state, showing a high dependence on threonine catabolism, in particular (Wang et al., 2009). Marked differences in the differentiation propensity exist among human ES (hES) cell lines and form an obstacle for the directed differentiation of hES/iPS cells *in vitro*. Here, we identified a specific dependence of the pluripotent human ES/iPS cells on a particular amino acid for their growth. In undifferentiated ES/iPS cells, a high expression level of enzymes participating in the particular amino acid cycle is observed, which rapidly decreased during differentiation into the definitive endoderm. We have established a differentiation procedure for human ES/iPS cells to differentiate into the definitive endoderm derivatives of the pancreas and liver (Shiraki et al., 2008, 2011). To establish a more endoderm selective differentiation method, we focused on the differences of amino acid metabolisms between the undifferentiated and differentiated definitive endoderm cells. When cultured in media deprived of the particular amino acid, undifferentiated human ES/iPS cells rapidly ceased to proliferate and apoptosis occurred, which resulted in an increased proportion of Sox17-positive definitive endoderm cells. Further differentiation into hepatic lineages was potentiated, to yield a 2-fold increase in Albumin secretion and 5-fold increase in Cyp3A4 activity. Moreover, the elimination of undifferentiated cells using the particular amino acid deprived media was applicable to several different human ES/iPS cell lines. In this study, a novel human ES/iPS cell differentiation system, based on the difference of a specific amino acid metabolism between undifferentiated cells and differentiated cells, is established. By using the media deprived of a particular amino acid, undifferentiated cells can be specifically eliminated during endoderm differentiation. This method is useful for directing human ES/iPS cell lines that are resistant to differentiation to achieve a higher differentiation efficiency.

Poster Board Number: F-3064

CONTRIBUTION OF *DE NOVO* DNA METHYLTRANSFERASES IN HUMAN EMBRYONIC STEM CELL SIGNATURE

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DNA methylation is a critical epigenetic modification that has important roles in development, cellular differentiation, genome regulation, and disease. Pluripotent stem cells possess a unique DNA methylation profile that is distinct from the profiles of all somatic cell types, and it is likely that the differences between the DNA methylation patterns of pluripotent and somatic cells contribute to the maintenance of the pluripotent state and/or commitment to differentiation. In humans, DNA methylation is placed by three DNA methyltransferases (DNMTs): The *de novo* DNA methyltransferases, DNMT3A and DNMT3B, have the ability to methylate previously unmethylated sequences, whereas the maintenance DNA methyltransferase, DNMT1, catalyzes methylation of the unmethylated cytosine in hemimethylated CpG dinucleotides, thus preserving the pattern of CpG methylation during cycles of DNA replication. DNMT3L (DNMT3-like) lacks enzymatic activity, but is known to be physically associated with DNMT3A and DNMT3B and increases their enzymatic activities and modulates their target sequence preferences. Our previous studies comparing human embryonic stem cells (hESCs), their differentiated derivatives, and differentiated primary cells have shown that the level of DNA methylation is inversely correlated with differentiation status. The highest level of methylation was found in the undifferentiated hESCs while the lowest was seen in the fully differentiated fibroblasts. A significant percentage of the DNA methylation in hESCs was observed to be at CpA dinucleotides, rather than canonical CpG dinucleotides. Using human pluripotent stem cells in which we have stably knocked-down DNMT3A and DNMT3B, we have assessed the contributions of DNMT3A and DNMT3B on the regulation of both CpG and non-CpG methylation in human pluripotent stem cells and their differentiated derivatives, and determined that DNMT3B is responsible for the bulk of non-CpG methylation, but also plays a role in normal CpG methylation. We have also used these cells to explore the roles of these enzymes in differentiation. By deciphering how epigenetic modifications are established, maintained, and modified in the pluripotent state and during differentiation, and how they are influenced by the cells' environment, may enable us to more effectively direct cellular differentiation and produce homogeneous cell populations of desired phenotypes that can be used as in vitro models for development and disease, as well as for drug development and cell therapy.

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MODULATION OF SOX17 AND HNF4A OVER-EXPRESSION ALTERS ANTERIOR-POSTERIOR ENDODERM FATE DURING HUMAN EMBRYONIC STEM CELL (HESC) DIFFERENTIATION

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hESC-derived definitive endoderm (DE) tissues such as liver and intestine are of great interest in regenerative medicine however, the transcriptional regulatory networks that orchestrate DE differentiation are poorly understood. We have previously shown that forced expression of SOX17 in hESCs specifies a distinct anterior DE progenitor population, providing an in vitro model to elucidate the regulatory networks driving DE specification and differentiation. *In vivo*, Sox17 expression is high at the onset of DE specification however, as the embryo undergoes morphological changes resulting in DE differentiation into the primitive gut tube, Sox17 expression shifts from the foregut to the mid- and hindgut regions. As development proceeds, the primitive gut tube is divided into organ domains marked by organ-specific transcription factor expression. The nuclear receptor transcription factor HNF4α is expressed in the liver, pancreas, stomach and intestine throughout organogenesis and is important for differentiation and maturation of these DE-derived cell types. In fibroblasts, HNF4α over-expression induces intestinal gene expression. The aim of this work is to combine SOX17 and HNF4α over-expression in hESCs to investigate the molecular regulation of DE cell fate restriction. A dual-inducible over-expression system was created using a PiggyBac transposon containing the tetO2 tetracycline/doxycycline inducible promoter and an ERT2 fusion protein to over-express HNF4α and SOX17, respectively. SOX17 alone, HNF4α alone or SOX17 and HNF4α together were induced for 2, 3 or 4 days. Gene and protein expression profiling of SOX17-hESCs reveals an induction of mesendoderm markers including CXCR4, DLX5, MIXL1 and FLK1, and maintenance of the pluripotency and anterior primitive gut tube marker SOX2, consistent with anterior DE identity *in vivo*. Interestingly, SOX17-hESCs maintain expression of pluripotency factors OCT4 and NANOG. In contrast, HNF4α-hESCs and HNF4α-SOX17-hESCs induce the HNF4α intestinal target CDX2 as well as PDX1 and HNF6, consistent with posterior gut identity *in vivo*. HNF4α-hESCs and HNF4α-SOX17-hESCs shut down SOX2, OCT4 and NANOG expression by day 4. SOX17 and HNF4α alone or in combination do not express extraembryonic endoderm genes SOX7, LAMB1 and AFP. Stable DE generated by forced expression of SOX17 for 6 days subsequently acquires posterior gut identity following forced HNF4α expression for 4 days. To determine whether HNF4α can alter anterior gut tube fate, HNF4α transgene levels were modulated during hepatocyte differentiation. HNF4α was induced at three stages of hepatocyte differentiation, DE specification, hepatic specification and hepatic maturation. Induction of HNF4α following DE specification results in inefficient hepatic differentiation and induction of the HNF4α intestinal target CDX2. HNF4α induction following hepatic specification or maturation enhances hepatic differentiation, demonstrated by a significant increase in ALB, CYP1A2 and CYP3A7, decreased AFP expression. HNF4α intestinal target genes are not induced. These experiments demonstrate that the temporal regulation of SOX17 and HNF4α during hESC differentiation can alter anterior-posterior DE fate. Future experiments will focus on elucidating transcriptional regulatory interactions during anterior-posterior DE specification.

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DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS IN A FOUR-COMPARTMENT HOLLOW-FIBRE BIOREACTOR PROMOTES HEPATOCYTE DIFFERENTIATION AND MATURATION

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There is a huge need for an unlimited supply of human hepatocytes, for use in drug discovery and toxicity studies. Today human primary hepatocytes (hPH) and animal models are used, but these model systems are marred by several disadvantages. The availability of good hPH is much restricted and experiments in fresh hPH can not be reproduced. Animal experiments are costly, have low throughput, and have limited human relevance. An in vitro model system, based on human stem cells, provides a stable and unlimited supply of human hepatocytes where the experiments can be reproduced in the long run using the same source. Human embryonic stem cells (hESC) are a very intriguing, pluripotent cell type with the capability of self-renewal. In recent years, much effort has been made to direct hESC towards the hepatic lineage with encouraging results. However, the aim of generating cells that are fully functional, comparable to human primary hepatocytes, have not yet been met, and hESC-derived hepatocytes have demonstrated reduced hepatic functionality. Therefore, further improvement of the differentiation process is needed and likely requires more sophisticated culturing systems. A three-dimensional (3D) arrangement of cells is the basis for hepatocyte polarity and functionality, while hPH placed in two-dimensional (2D) cultures, rapidly loose much of their hepatic functionality and are thus not useful for studies of e.g. drug metabolism and toxicity. The present study investigates if hepatic differentiation of hESC can be improved by using a 3D bioreactor culture system that better mimics the *in vivo* situation. While the 2D culturing system offers static conditions with discontinuous medium and gas exchange only via diffusion, the bioreactor technology offers a dynamic perfusion culture. A special four-compartment hollow-fibre bioreactor has been used that mimic the blood flow in the liver by decentralized nutrient and gas supply to the cells with low gradients. Parallel differentiation experiments were performed in both 2D and 3D culturing systems, and cells were sampled at four time points (d0, d7, d12, and d26). Typical hepatocyte markers were evaluated using qRT-PCR and immunohistochemistry. Moreover, whole genome gene arrays were applied to examine the global expression profile across the two differentiation regimes. Interestingly, our results show significant differences in gene expression patterns between the two systems. Using a combined criteria of t-test with p-value<0.05 and fold change<2 we identified 102 up-regulated and 63 down-regulated genes when the hepatocyte differentiation was performed in the 3D bioreactor, compared to the 2D system. We also screened for pathways that were affected by the bioreactor differentiation system and identified 10 KEGG-pathways that where significantly up-regulated. Interestingly, these pathways are highly related to liver functions, supporting the hypothesis that this 3D culturing system promotes hepatic differentiation. Moreover, in total 19 KEGG pathways were down-regulated during 3D differentiation. Notably, many of these repressed pathways are associated with apoptosis and cell proliferation, indicating that the 3D differentiation may

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stimulate maturation processes in the cells. Taken together, our results suggest that hepatic differentiation of hESC is improved when using this 3D bioreactor technology.

Poster Board Number: F-3067

BIFUNCTIONAL MIR-181A/A*, AND THE NEURAL-ASSOCIATED MIR-125B AND MIR-124 IMPACT ON SUBSPECIFICATION OF HUMAN NEURAL STEM CELL-DERIVED NEURONS

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During neural development, miRNAs control various aspects of neurogenesis ranging from neural stem cell self-renewal to differentiation into functional neurons. However, despite the high numbers of miRNAs expressed in the central nervous system, their roles in the development of specific neuronal subtypes remain largely unknown. Here, we made use of our recently established human embryonic stem cell-derived neuroepithelial-like stem cells (lt-NES) to identify novel miRNAs associated with human neuron differentiation and fate specification. By gain- and loss-of-function analyses we show that miR-181a/a* and the known brain-enriched miR-124 and miR-125b are required for the differentiation of lt-NES cells into neurons. Lt-NES cells differentiate mainly into GABAergic interneurons, but can also be instructed to generate dopaminergic and serotonergic phenotypes. We therefore assessed the impact of these miRNAs on neuronal fate specification. Our data demonstrate that miR-181a and miR-125b promote, while miR-181a* and miR-124 inhibit the generation of dopaminergic neurons in differentiating lt-NES cell cultures. These data depict bifunctional miR-181a/a* as well as miR-124 and miR-125b as novel regulators of human neural stem cell differentiation and as biomedical tools for modulating subspecification of stem cell-derived neurons.

Poster Board Number: F-3068

DIRECTING THE DIFFERENTIATION OF HUMAN ES CELLS TOWARDS A RENAL FATE.

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The goal of our project is to direct differentiation of pluripotent human ES cells to a renal progenitor state. During embryogenesis, the inner cell mass develops into kidney progenitors via two key intermediate stages, mesendoderm and intermediate mesoderm. It has previously been shown that mesendoderm differentiation can be induced via the addition of BMP4 and ActivinA with the relative ratios of these factors determining the end of the primitive streak being induced (Tam PP, 2007). To find the optimum ratio of BMP4/Activin A for mesoderm induction, human ES cells were cultured with different combinations of BMP4/Activin A, including 30/0, 30/10, 20/100 and 5/200 (ng/mL). We have previously generated a MIXL1GFP/wt Hes3 cell line (Davis et al, 2008) to detect GFP as a readout of mesendoderm differentiation. In all three combinations of 30/10, 20/100 and 5/200, >80% of cells expressed GFP as assessed by FACS, indicating mesendoderm induction. However, by qPCR analysis, highest expression of T (Brachury), a mesoderm marker, was detected in the combination of 30/10, whereas highest expression of SOX17, an endoderm marker, was detected in the 5/200. In addition, formation of embryoid bodies followed by spontaneous differentiation for a further 10 days resulted in the induction of the trunk mesoderm marker, OSR1, only after 30/10 BMP4/Activin A. These results indicate that high BMP4 and low Activin A bias towards induction of posterior primitive streak, contributing to mesoderm formation, while low BMP4 and high Activin A induce anterior primitive streak, contributing to endoderm. Next, we directed the differentiation from posterior primitive streak to intermediate mesoderm using several defined growth factors known to be involved in intermediate mesoderm and metanephric mesenchyme development *in vivo*. Without these growth factors, the cells differentiated to only OSR1 positive trunk mesoderm. However, with the growth factors, the induced cells expressed other intermediate markers, PAX2 and LHX1 as well as OSR1. Furthermore, by IF analysis, OSR1 and LHX1 were detected in the same cells that expressed PAX2, indicating OSR1, PAX2 and LHX1 triple positive intermediate mesoderm cells were induced. Finally, as we are seeking to improve the specificity of this differentiation, we have generated a new double fluorescent reporter line (CRYMCerulean/wt; MIXL1GFP/wt line). In this cell line, the cyan fluorescent protein Cerulean is knocked into the nephron progenitor marker CRYM gene locus so that the cells will turn blue if differentiating into the nephron progenitor stage. Using stepwise differentiation, starting from BMP4/Activin A for mesendoderm induction followed by the defined factors for intermediate mesoderm then nephron progenitor inductions, we observed Cerulean positive cells by day 10 of differentiation. At this time, cells immunopositive for the nephron progenitor protein SIX2 were also detected. Hence, the use of this double targeted cell line will make it feasible to enrich for nephron progenitors using FACS as well as facilitating the optimization of differentiation culture conditions using either known growth factor combinations or by screening chemical compound libraries.

Poster Board Number: F-3069

FUNCTIONAL NEURONAL CELLS GENERATED BY HUMAN PARTHENOGENETIC STEM CELLS

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In the brain large numbers of genes are imprinted and imprinting plays non-redundant and reciprocal roles in brain development. This could indicate that human parthenogenetic embryonic stem cells (hpESCs) lacking the paternal genome have limited neural potential. As the neural developmental potential of hpESCs was not assessed so far, we determined the abilities of hpESCs to differentiate into various neural subtypes and examined the DNA methylation and expression states of imprinted genes in hpESCs under neural differentiation. In hpESC-derived neural stem cells (hpNSCs) we observed upregulation of NSC markers Sox1, Nestin, Pax6, and Musashi1, silencing of pluripotency marker genes (Oct4 or Nanog) and absence of activation of neural crest (Snai2, FoxD3) and mesodermal (Acta1) markers. Immmunocytochemial anaylses revealed that hpNSCs cultures ubiquitously expressed NSC markers Nestin, Sox1, Sox2 and Vimentin. Upon differentiation for 28 days phNSCs generated neural subtypes with specific neural morphology and expression of neuronal (Tuj1, NeuN, Map2, Tau, Synapsin1

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and GABA) and glial markers (GFAP, O4). phNSCs were responsive to instructive regionalization cues and differentiated into regional phenotypes such as midbrain dopaminergic and motoneurons. Electrical stimulation elicited multiple action potentials with a maximum frequency of 30 Hz in hpNSC-derived neuron-like cells and the cells depicted a typical neuron-like Na+/K+ currents that responded to pharmacological blockers of sodium (tetrodotoxin) and potassium (tetraethylammonium) channels. Further we observed that in hpESCs and hpNSCs the majority of CpGs of KvDMR1 were methylated and DMR1 (H19/Igf2 locus) exhibited partial or complete absence of CpG methylation, which is consistent with a parthenogenetic origin. Imprinted gene expression analyses showed that upon differentiation parent of origin-specific expression is maintained in hpESCs and hpNSCs. Our results demonstrate that despite the lack of a paternal genome, hpESCs generate proliferating NSCs that are capable of differentiation into physiologically functional neuron-like cells and maintain allele-specific expression of imprinted genes. Thus, hpESCs could have important implications for studies of the role of maternal and paternal genomes on neural development and to better understand imprinting-associated brain diseases.

Poster Board Number: F-3070

GENERATION OF METABOLICALLY FUNCTIONING HEPATOCYTES FROM HUMAN EMBRYONIC STEM CELLS AND INDUCED PLURIPOTENT STEM CELLS BY TRANSDUCTION OF FOXA2 AND HNF1A

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Hepatocyte-like cells differentiated from human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) are expected to be utilized as a tool for screening for hepatotoxicity in the early phase of pharmaceutical development. Although we have recently reported that hepatic differentiation is promoted by sequential transduction of SOX17, HEX, and HNF4α into hESC- or hiPSC-derived cells (*Mol Ther*. 2012 Jan; 20(1): 127-37), further maturation of the hepatocyte-like cells is required for widespread use of drug screening. To screen for hepatic differentiation-promoting factors, we selected the seven candidate genes (FOXA2, HEX, HNF1α, HNF1β, HNF4α, HNF6, and SOX17) that are related to the liver development. We identified a combination of two transcription factors, comprising FOXA2 plus HNF1α, which could promote efficient hepatic differentiation from hESCs and hiPSCs. The gene expression profile of hepatocyte-related markers (such as cytochrome P450 enzymes, conjugating enzymes, hepatic transporters, and hepatic nuclear receptors) in the hepatocyte-like cells was comparable to that in primary human hepatocytes that were cultured for 48 hr after the cells were plating. The hepatocyte-like cells had various hepatic functions: ability of albumin and urea secretion, and uptake of indocyanine green and low density lipoprotein. Experiments to estimate the drug metabolism capacity by measuring the metabolites of nine drugs (Phenacetin, Bupropion, Paclitazel, Tolbtamide, S-mephenytoin, Bufuralol, Midazolam, Testosterone, and Hydroxyl coumarin) indicated that our hepatocyte-like cells had the capacity to metabolize all nine drugs. Furthermore, the hepatocyte-like cells could catalyze the toxication of Benzbromarone. Our technology, which utilizes transduction of FOXA2 and HNF1α, would be a useful tool for the efficient generation of metabolically functioning hepatocytes from hESCs and hiPSCs. Moreover, the hepatocyte-like cells would be a valuable tool to predict drug toxicity as well as primary human hepatocytes.

Poster Board Number: F-3071

TYPE I COLLAGEN PROMOTES HEPATIC MATURATION FROM HUMAN PLURIPOTENT STEM CELLS IN 3D CO-CULTURE WITH SWISS 3T3 CELL SHEET.

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Recently, several studies have shown that human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) can differentiate into hepatocyte-like cells. Although primary human hepatocytes are generally employed in drug screening, these cells have disadvantages in the availability of sources, variability from batch-to-batch, and de-differentiation capacity. Because hESC- or hiPSC-derived hepatocyte-like cells (hEHs or hiPHs, respectively) have potential to resolve these problems, they are expected to be applicable in drug screening. Recently, we have established the efficient methods for hepatic differentiation from hESCs and hiPSCs by sequential transduction of FOXA2 and HNF1a. Although this study showed that the hEHs and hiPHs expressed the similar gene expression levels of hepatocyte-related markers as compared with those of primary human hepatocytes and they could metabolize various types of drugs, further hepatic maturation is required for wide-spread use of drug screening. It is known that primary hepatocytes maintained their functions for a long time by the three-dimensional (3D) co-culture, the combination of 3D culture and co-culture. Although 3D culture or co-culture methods are each applied to promote hepatic differentiation from hESCs or hiPSCs, only a few studies have investigated the hepatic differentiation from hESCs or hiPSCs using a 3D co-culture method. In this study, we utilized a cell sheet engineering technology to promote hepatic maturation. By 3D co-culturing of the hEHs and hiPHs with Swiss 3T3 cell sheet, the gene expression levels of hepatocyte-related markers were significantly increased in comparison with those in the hEHs and hiPHs cultured as monolayer. The amount of ALB secretion was also increased in the hEHs and hiPHs by 3D co-culture with Swiss 3T3 cell sheet. These data indicated that stratification with Swiss 3T3 cell sheet promoted the hepatic maturation of the hEHs and hiPHs, and that Swiss 3T3 cells produces hepatic maturation factors. To elucidate Swiss 3T3 cell-derived hepatic maturation factors, the hEHs were cultured in cell culture-insert systems or co-cultured with Swiss 3T3 cells. The results showed that enhanced hepatocyte-related gene expression was observed only in co-culture condition with Swiss 3T3 cells, indicating that physical contacts between the hEHs and Swiss 3T3 cells play an important role in hepatic maturation of the hEHs. We further investigated the role of type I collagen, which is abundantly synthesized in Swiss 3T3 cells, in hepatic maturation, because extracellular matrices are known for the production of essential factors for hepatic differentiation. Showing the same result as Swiss 3T3 cell sheet, the hEHs stratified

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with type I collagen gel also significantly elevated the expression of hepatocyte-related genes as compared with the hEHs. We also found that Swiss 3T3 cell-mediated hepatic maturation of the hEHs was suppressed by the treatment of a collagen synthase inhibitor. Taken together, these results showed that hepatic maturation from hESCs and hiPSCs was promoted by 3D co-culture with Swiss3T3, and this effect is largely mediated by Swiss 3T3 cell-derived type I collagen. The 3D co-culture with Swiss 3T3 cell sheet should be effective to promote maturation of the hepatocyte-like cells derived from pluripotent stem cells. Our method would be a powerful tool for medical applications, such as drug screening.

Poster Board Number: F-3072

NEURONS DERIVED FROM FRAGILE X HUMAN EMBRYONIC STEM CELLS EXPRESS RETARDED DIFFERENTIATION IN-VITRO

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Fragile-X Syndrome (FXS) is the most common form of inherited mental retardation, caused by developmentally regulated inactivation of FMR1, leading to the absence of its protein FMRP. We have previously shown that undifferentiated Fragile-X human Embryonic Stem Cells (FX-hESCs) express FMRP, albeit the presence of the full FMR1 mutation. This unique feature of FXS and FX-hESCs is not recapitulated by FMR1 knock-out animals and FX-human induced pluripotent stem cells which do not express FMRP even at early stages of development/differentiation. We report here, for the first time, in-vitro differentiation of three different FX-hESC lines into mature neurons, progressively inactivating FMR1. Abnormal neurogenesis and aberrant gene expression were found already at the early stages of differentiation, leading to poor neuronal maturation and high gliogenic potential. Human FX-neurons were electrophysiologically functional and contained the appropriate molecular synaptic machinery. However, they displayed poor spontaneous synaptic activity and lacked reactivity to glutamate. Our dynamic FX-hESCs model is expected to contribute considerably to the understanding of the mechanisms by which neurogenesis is altered in FXS individuals, leading to mental retardation. Furthermore, it is a suitable platform for advanced drug screening and development of new therapeutic strategies for FXS.

Poster Board Number: F-3073

RECURRENT VARIATIONS IN DNA METHYLATION IN HUMAN PLURIPOTENT STEM CELLS AND THEIR DIFFERENTIATED DERIVATIVES

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In this study, we explored epigenetic and transcriptional variation in the most comprehensive collection of hPSC and somatic samples to date. Using a combination of genome-wide DNA methylation and mRNA expression data, we identified unique epigenetic and transcriptional properties of the pluripotent state. Most distinctive among these characteristics were prevalent, but not uniform, losses of imprinting and X chromosome inactivation (XCI) and consistent hypermethylation of somatic cell-type-specific genes in hPSCs. We observed the acquisition of appropriate cell-type-specific DNA methylation marks during differentiation of hPSCs, despite persistence of aberrant imprinting and XCI. In order to determine which imprinted genes we could confidently analyze in our study, we identified a panel of loci that showed appropriate imprinting in normal tissue samples, as well as reciprocal methylation in gynogenetic and androgenetic samples. We observed aberrations at many of the examined imprinted genes in a substantial subset of hPSCs; changes at some loci arose during reprogramming and others over time in culture. Using linear regression, we were able to correlate the imprinting status of some imprinted genes with specific *in vitro* manipulations, while the status of the other imprinted genes in our study were independent of the identifiable variables. We observed a large degree of variability in X chromosome CpG methylation in female hPSCs, which was dependent on the level of XIST expression. Our results were consistent with a loss of XIST expression with time in culture, followed by erosion of DNA methylation, originating in several sub-segments of the X chromosome and spreading to involve larger regions. Recurrent loss of XCI was observed in 49 X-linked disease genes, meriting caution in the use of female hPSCs for studies of X-linked disease. We found that DNA hypomethylation was the most discriminate epigenetic feature in all of the 17 tissue types analyzed, and that tissue-specific hypomethylated genes are associated with the function of that tissue. We observed DNA methylation changes during the directed differentiation of hPSCs into neural progenitor cells and oligodendrocyte precursor cells that recapitulated patterns of DNA methylation at neural and oligodendrocyte-specific genes in fetal and adult brain samples, suggesting that an interplay between DNA methylation and demethylation regulates cellular differentiation. Among the tissue-specific hypomethylated genes were transcription factors used for the transdifferentiation of fibroblasts into neurons, master regula-

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tors of oligodendrocyte differentiation, and iPSC reprogramming factors. We suggest that the identification of uniquely hypomethylated genes will permit the discovery of high-level regulators of cellular identity, and may inform the selection of factors for novel transdifferentiation protocols. Additionally the recapitulation of unique patterns of DNA methylation in directed differentiation supports the validity of hPSCs as models of development and disease.

Poster Board Number: F-3074

STABLE EXPRESSION OF GFP IN ENGINEERED HUMAN EMBRYONIC STEM CELL LINES DURING HEPATOCYTES DIFFERENTIATION

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The embryology of the liver has been studied during the past decades using different animal models including rodents, zebra fish, and chicken. Though the growth factors involved in hepatic differentiation have been characterized, little is known about the transcription factors and signaling pathways that play a key role in cell fate commitment. Also, some results are contradictory, depending on what models have been used. Not only do the few cells present in developing organs represent a challenge for genome-wide analyses, but also specific molecular mechanisms are likely to vary between species. As a result, it has become important to study the development of the human liver using a species-specific model. Since human embryonic stem cells (hESCs) can be directed *in vitro* to differentiate into hepatocyte-like cells in a staged fashion that mirrors the steps that occur during normal hepatogenesis, they represent such a model. Reliable and efficient genetic manipulation methods, including those for marking cells of interest, are key tools for the study of gene function in hESCs. Random integration of marker genes by viral or non-viral-mediated delivery lacks control over copy number, the site of integration and gene expression level. Here, we have taken advantage of a well-established hESC line that expresses GFP in a constitutive manner, in both the undifferentiated and differentiated state. The CAG promoter-driven GFP cassette is specifically integrated into a predetermined genomic locus at chromosome 13q32.3. To ensure constant transgene expression, a cHS4 double insulator fragment is designed to flank the GFP transgene. The hESCs generated express GFP constitutively (~100%) in long term culture on feeders or feeder free conditions. Here, we explore the potential of this GFP-hESC line to differentiate into functional hepatocytes using fully defined culture media devoid of animal products or unknown factors. This approach recapitulates development by re-enacting key stages of liver development. We believe that this is the best approach for generating differentiated liver cells with native properties. The early hepatoblasts generated coexpress AFP, CK19, and HNF4. These cells further express many mature hepatocyte-specific genes including albumin, tyrosine aminotransferase, tryptophan oxygenase, alpha-1-antitrypsin, and the asialoglycoprotein receptor after a period of culture in maturation conditions. They secrete albumin, are capable of internalizing DiL, and can store glycogen. Most importantly, the differentiated cells still express GFP (>90%), and therefore can be tracked in transplantation or co-culture experiments. We have shown here that targeting the 13q32.3 locus leads to stable and long term expression of a transgene during the course of directed *in vitro* differentiation. In addition, this method can be applied in future studies to generate hepatic lineage-specific reporters, which will also be useful for studies to improve the directed differentiation process, and facilitate the study of liver development using hESC-based *in vitro* models.

Poster Board Number: F-3075

EFFECTS OF AHR AGONIST ON NEURONAL CELL DIFFERENTIATION FROM HUMAN ES CELLS

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In the health risk research, a new *in vitro* system which can investigate effects of chemical exposures during early stages of development of human embryo on cellular function or individual phenotype at the later stages are now required. Arylhydrocarbon receptor (Ahr) is one of the nuclear receptors that mediate the response to xenobiotics like polyaromatic hydrocarbons and dioxins. Activation of this receptor in the embryo causes teratogenesis or many types of adverse effects. Here we report the effects of Ahr activation by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on neuronal cell differentiation from hESCs. Embryoid bodies generated from KhES1 cells were plated on matrix-coated plates to differentiate neuronal cells. TCDD (0.1, 1, 10 nM) was exposed to ES cells on Day0, EBs on Day9, and neuronal cells on Day35 for 24 hrs, respectively. The mRNA induction of CYP1A1, a biomarker of dioxin exposure, was detected only in the Day9-exposed samples. Clear neuronal cell differentiations were observed at Day40 in all exposure groups, indicating no inhibitory effects of TCDD. However, in the Day9-exposed groups, the rate of neural rosette formation was increased by TCDD (1 nM and 10 nM). TCDD exposure on Day9 also enhanced NESTIN and MTAP2, but decreased SOX17 and FOXA2 on the later stage of culture. These results indicated that late stage of EBs were more sensitive to TCDD than other stages examined. The higher sensitivity may be due to the following inhibition of differentiation of endoderm cells and leads the higher rate of differentiation of ectoderm cells. The research was supported by Grant-in-aid from the Ministry of Health, Labour and Welfare of Japan.

Poster Board Number: F-3076

NEGATIVE REGULATION OF ERYTHROID PROGENITOR PROLIFERATION BY TGF BETA SIGNALING IN HUMAN PLURIPOTENT STEM CELLS

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Patient-specific human induced-pluripotent stem cells (hiPSCs) represent an important cell sources to treat patients with acquired blood disorders. To realize the therapeutic potential of hiPSCs, it is crucial to understand signals that direct hiPSC differentiation to a hematopoietic lineage fate. Although our primary goal is to assess the erythroid cell generation from hiPSCs, human embryonic stem cells (hESCs) remain a "gold standard" for studies of pluripotent stem cells. Our previous study demonstrated that hESC-derived $CD34^+$ cells contain progenitors to give rise to hematopoietic cells, endothelial cells and smooth muscle cells, suggesting that $CD34^+$ cells are heterogeneous population. To identify the signals that regulate the differentiation of hESC- and hiPSC-derived $CD34^+$ hematopoietic progenitors to erythroid cells, we have established

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a serum-free and feeder-free differentiation system. We examined the effect of hematopoietic growth factors on lineage differentiation of CD34+ hematopoietic progenitors, and found that EPO signaling had a dominant effect on erythroid differentiation. An addition of EPO significantly increased the number of cells that expressed erythroid markers, CD71 and CD235a (Glycophorin A), and elevated the hemoglobin gene expression, indicating that EPO signaling promotes erythroid progenitor proliferation and maturation. Transforming growth factor beta (TGF-beta) is a key regulator of hematopoiesis with potent inhibitory effects on progenitor and stem cell proliferation. TGF-beta receptor I and II were expressed in CD34+ hematopoietic progenitors, their gene expressions were gradually decreased during erythroid differentiation. Despite the presence of EPO and other hematopoietic growth factors, an addition of TGF-beta 1 significantly decreased the number of cells and the number of BFU-E colonies, while the frequency of CD71+ cells was unchanged. To further investigate whether inhibition of TGF-beta signaling affects CD34+ hematopoietic progenitors to erythroid cells, we added TGF-beta inhibitor, SB431542, at different stages of erythroid differentiation. Our data demonstrate that inhibition of TGF-beta signaling at early stage of promoted erythroid progenitor proliferation, while an addition of TGF-beta inhibitor at late time points had less profound effect on erythroid differentiation. Our study suggested that inhibition of TGF-beta synergizes EPO signaling to promote erythroid progenitor proliferation, but has less (or no) impact on EPO-mediated erythroid maturation.

Poster Board Number: F-3077

INHIBITION OF TGF-BETA SIGNALING IN THE EARLY MESODERM STAGE PROMOTES THE DEVELOPMENT OF HEMATOPOIETIC PROGENITORS FROM HUMAN EMBRYONIC STEM CELLS

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The clinical potential of human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) for regenerative medicine should be realized when we find a way to manipulate tissue-specific differentiation more efficiently. Among the various cell types that can be derived from pluripotent stem cells (PSCs) in culture, the hematopoietic lineages in particular have great clinical applications, such as transfusion therapies (erythrocytes and platelets), hematopoietic cell transplantation (hematopoietic stem cells, HSCs), and cell-based immunotherapy (immune cells). For the above applications of PSC-derived cells to be realized, solid strategies need to be developed that promote the efficient and reproducible differentiation of both ESCs and iPSCs into the hematopoietic lineages. In order to achieve this goal, we first performed chemical compound screening using *in vitro* differentiation system of human ESC based on embryoid body (EB) formation. From this screen, we found that several TGFβ inhibitors enhanced hematopoietic cell differentiation. To further study this effect, we evaluated the role of TGFβ signaling pathway on the induction of hematopoietic cells by measuring the emergence of CD34+CD43+CD45+ hematopoietic progenitor cells (HPCs) and their colony formation ability. We found that treatment with the TGFβ inhibitor, LY364947 on the early mesoderm stage, followed by EB differentiation at day 4 to 6 significantly increases the proportion and number of hematopoietic progenitors at EB day 8, resulting in approximately 2-fold higher induction of HPCs compared to EBs without treatment. Of interest, treatment of EBs at the late mesoderm stage (after EB day 6) had no effect on the induction of HPCs. HPCs treated with LY364947 showed indistinguishable characteristics from the control HPCs in colony-forming capacity as well as gene expression profiles detected by microarray analysis. In contrast, gene ontology (GO) analysis of KDR+CD34+ early mesodermal cells from EB culture at day 6 revealed that LY364947 treatment on EBs at day 4 to 6 enhances expression of gene sets related to vasculogenesis, signal transducer activity, and development. Our findings indicate that the TGFβ signaling pathway negatively regulates hematopoietic development at the early mesoderm stage during human ESC differentiation. These findings demonstrate the importance of elucidating the role of critical signaling pathways before the emergence of the hematopoietic cells and provide the basis for the development of more advanced strategies to direct the differentiation of human ESCs to HPCs.

Poster Board Number: F-3078

DAP5 MEDIATED TRANSLATION CONTROL OF HUMAN EMBRYONIC STEM CELL DIFFERENTIATION

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Little is known about the translational mechanisms that regulate embryonic stem cell (ESC) pluripotency and differentiation, which can contribute to the proteome composition of the cell by directing the selective translation of critical mRNAs. Most of translation control occurs at the level of initiation. For example, in addition to cap dependent translation, which drives the majority of protein synthesis, there is an alternative translation initiation mechanism that is cap independent. This mechanism involves the recruitment of the ribosome directly to specific mRNAs that contain an Internal Ribosome Entry Site (IRES) on their 5'UTR, close to the initiation codon. DAP5 is an eIF4G homologue that mediates cap-independent translation, particularly under cell stress and apoptotic conditions when cap-dependent translation is compromised. Notably, it has been reported that DAP5 knock-out mice die at an early stage of gastrulation, and that the differentiation of DAP5 -/- mouse ESCs is impaired. This most likely stems from a critical DAP5 function in mediating IRES-dependent translation of specific target mRNAs that are necessary for differentiation of ESCs. To test this hypothesis, we have generated stable human ESC (H9) expressing control or DAP5-targeting shRNA to successfully knock-down (KD) DAP5 expression. DAP5 KD cells showed normal hESC morphology and no obvious differences in proliferation rate was detected. However, DAP5 had a critical role in differentiation of the hESCs under retinoic acid (RA) treatment and spontaneous differentiation as embryoid bodies (EB). DAP5 depletion prevented very significantly the strong reduction in the expression of the pluripotent markers Nanog and Oct4 in 10-20 days EBs, and also in RA-treated hESCs, as compared to control cells. In addition, differentiation markers HoxA1 and RARβ showed reduced expression in DAP5 KD cells treated with RA. At the phenotypic level, DAP5 KD EBs were morphologically distinct from control EBs, remaining round and compact even after prolonged culturing periods. The frequency of apoptotic cells was increased in these EBs, and cell extracts displayed higher caspase-dependent activity and lower steady state levels of Bcl2, a known target of DAP5. These data suggest that DAP5 is critical for the differentiation of hESCs. We are currently looking for other DAP5 mRNA targets that play a role during stem cell differentiation.

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Poster Board Number: F-3079

DIRECTED DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS INTO THYMIC EPITHELIAL PROGENITORS

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The thymus plays a central role in the immune system as it is required for T cell differentiation and TCR repertoire selection. It removes self-reactive T cells and helps drive the production of important suppressor populations like regulatory T cells which are critical regulators of immune tolerance. One key population of cells residing in the thymus are thymic epithelial cells (TECs). These cells support and guide T cell development by imposing both positive and negative selection steps. Given their key role in supporting T cell development and controlling immune tolerance, the ability to generate TECs from pluripotent stem cells would have numerous applications. We have developed a novel method for generating thymic epithelial progenitors (TEPs) from human embryonic stem cells (hESCs). We have used molecules and growth factors known to guide TEC development during embryogenesis to direct hESCs towards the TEC lineage *in vitro*. Key developmental stages such as differentiation to definitive endoderm and pharyngeal endoderm are recapitulated, as shown by expression of the specific markers Sox17 and Foxa2 (definitive endoderm) as well as Hoxa3, Eya1 and Gcm2 (pharyngeal endoderm). Gene expression analysis showed that cells differentiated using this protocol expressed the transcription factor Foxn1, a critical regulator of TEC lineage development, in addition to other genes found in the developing thymus (Hoxa3, Eya1, Pax1). We are currently investigating if hESC-derived TEPs can support T cell maturation. If successful, this work will make possible the use of human pluripotent stem cells as a source of renewable thymic cells and a means to reinforce immune tolerance to hESC-derived transplants.

Poster Board Number: F-3080

DERIVATION, CULTURE AND RETINAL PIGMENT EPITHELIAL DIFFERENTIATION OF HESCS USING HUMAN FIBROBLAST FEEDER CELLS

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Retinal pigment epithelium cells derived from human ESCs could be useful for restoring retinal function in age-related macular degeneration. However the use of non-human feeder cells to support the growth of ESCs for clinical applications raises the concern of possible contamination because of direct contact between animal and human cells. In this study, we produced human ESCs using human fibroblast feeder layers isolated from foreskin and abdominal tissues. Using this system, human ESCs differentiated into retinal pigment epithelium cells in differentiation medium. Seven human ESC lines were established from 18 blastocysts. These human ESCs showed normal morphology, expressed all expected cell surface markers, had the ability to form embryoid bodies upon culture *in vitro* and teratomas after injection into SCID mice, and differentiated further into derivatives of all three germ layers. Under conditions of committed differentiation, these human ESCs could differentiate into retinal pigment epithelium cells after 2 months in culture. In conclusion, the results of this study demonstrated that human foreskin/abdominal fibroblasts have the potential to support the derivation and long-term culture of human ESCs, which can then be used to generate retinal pigment epithelium cells with characteristic morphology and molecular markers.This technique avoids the concerns of contamination from animal feeder layers during human ESC derivation, culture and differentiation, and will thus facilitate the development of retinal pigment epithelium cell transplantation therapy.<!--EndFragment-->

Poster Board Number: F-3081

WNT SIGNALLING ORCHESTRATES FIRST AND SECOND HEART FIELD DIFFERENTIATION FROM HUMAN EMBRYONIC STEM CELL DERIVED LATERAL PLATE MESODERM

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Chronic heart failure after myocardial infarction is a leading cause of premature death worldwide. Endogenous cardiomyocyte regeneration following such heart insult is insufficient to compensate for loss of injured tissue. However, evidence for some cardiomyocyte renewal in the adult human heart is encouraging. We are pursuing novel techniques to promote this regeneration, which may result in the development of novel therapies aimed at promoting the recovery of cardiac function after myocardial insult. Although the involvement of Wnt signalling in cardiogenesis is well established, the exact regulatory mechanisms whereby cardiomyocytes differentiate still have to be elucidated. Indeed, Wnt signalling was shown to be required for mesoderm formation while, at a later stage, its inhibition was found to be essential for the specification of cardiac precursors. Furthermore, there is evidence for an inhibitory role for canonical Wnt signalling in first heart field specification and a stimulatory role in the development of second heart field precursors. Human embryonic stem cells represent an ideal model system to investigate the application of small molecules in promoting heart differentiation and patterning. Treatment of H9 cells with bFGF, BMP4 and the PI3K inhibitor Ly294002 promoted differentiation of cardiogenic mesoderm in chemically defined conditions as indicated by the expression profile of GATA4, MEF2C, ISL1, FLK1 and NKX2.5 both with RT/QPCR and immunocytochemistry. This differentiation protocol was further optimised by modulating Wnt signalling with BIO, a Wnt agonist, and XAV939, a Wnt antagonist, to effectively differentiate cardiogenic mesoderm. Interestingly, treatment with XAV939 at a later stage promoted a second heart field profile of expression (NKX2.5, ISL1^{high}, MEF2C, HAND2, FGF10) in a dose dependent manner. Moreover, treatment of cardiogenic mesoderm with BIO appeared to elicit the opposite response stimulating a first heart field signature (TBX5, HAND1, ISL1^{low}, NKX2.5). Although these results appear to partially contradict our expectations, the discrepancies found could be explained by a multiphasic role for Wnt signalling during human cardiogenesis. Furthermore, these results elucidate how Wnt signalling orchestrates differentiation of lateral plate mesoderm to first and second heart field, thereby laying the foundation for the development of novel therapies directed at the functional recovery after cardiac insult.

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Poster Board Number: F-3082

MESODERMAL PROGENITOR GENERATION FROM HUMAN EMBRYONIC STEM CELLS AND INDUCED PLURIPOTENT STEM CELLS

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In vitro differentiation models of human embryonic stem cells (hESC) and human induced pluripotent stem cells (hIPSC) have become essential tools to understand dynamic developmental processes occurring during the human embryogenesis. While hESC-based assays have led to the identification of common ancestors to endothelial and hematopoietic cells, recent studies have also suggested the existence of common mesodermal precursors to mesenchymal, endothelial, and hematopoietic progenitors. However, the nature of such bipotent (mesenchymal/pericytic - endothelial) vasculogenic progenitors remains elusive. Various human mesodermal progenitors have been derived from pluripotent stem cells using diverse methodologies, but their existence and their hierarchy during development is unclear. Here we describe a hESC/hIPSC differentiation system to derive mesenchymal/pericyte progenitors concomitantly with endothelial and hematopoietic progenitors. Using a modified human embryoid body (hEB) system, various mesodermal progenitors were generated from hESC (H9), human fibroblast-derived IPSC (HUF1) and cord blood-derived IPSC (CB-6.2) under feeder-free endothelial culture conditions. After 4-5 days of differentiation in angiogenic conditions, distinct cell populations were sorted by flow cytometry based on the expression of CD146 and CD31. Using mesenchymal stem cell (MSC) culture conditions, CD146+CD31- cells were able to expand rapidly as a fibroblastic monolayer, expressing MSC (CD105, CD73, CD90) and pericytic (CD146, PDGFRb) markers. These populations lacked endothelial, hematopoietic or hemangioblastic (CD31, CD34, CD45, CD143/BB9) markers. Conversely, while all cell populations could be further expanded in endothelial conditions, only the CD31+CD146+ and CD31+CD146- fractions were able to give rise to a population of cells expressing endothelial cell markers (CD31, CD34, CD73, CD105, CD146). Simultaneous with the appearance of pericyte-like and endothelial cells, a large amount of multipotent CD34+CD45+ hematopoietic progenitors emerged directly from these adherent endothelial/stromal layers. This hEB-based mesodermal differentiation system provides a robust and optimized method for direct time course studies of mesodermal lineage commitment events. We are currently characterizing hESC/hIPSC-derived pericyte-like mesenchymal progenitors for their differentiation potential towards multiple mesenchymal lineages, including their potential for cardiomyogenesis. Additionally, we will further explore the kinetics of divergence between the various mesodermal lineages to clarify the hierarchy between each population.

Poster Board Number: F-3083

RESVERATROL AMELIORATES THE MATURATION PROCESS OF AN OPTIMIZED DIFFERENTIATION PROTOCOL OF HUMAN EMBRYONIC STEM CELLS TO B-CELL-LIKE CELLS.

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Embryonic Stem Cells (ESCs) have the capacity to differentiate into many cell types. Several studies have already demonstrated that it is possible to direct the differentiation of hESCs to the β-pancreatic lineage. However, the low efficiency and reproducibility of insulin-secreting cells production and the low amount of insulin secreted by these cells, indicate that we are still a step away from the achievement of a fully differentiated phenotype. On the other hand, recent studies in both insulinoma cell lines and human pancreatic islets, showed the impact of Resveratrol (RSV) on insulin secretion even though the mechanism by which this polyphenol potentiates glucose-stimulated insulin secretion is still not clear. The aim of this study was to optimize an efficient differentiation protocol that mimics *in vivo* pancreatic organogenesis by directing human ESCs through stages like definitive endoderm, pancreatic endoderm and endocrine precursor and to investigate the effect of RSV at the final steps of the differentiation process for further in vitro maturation. Our results indicated that treatment of HS-181, a human ESCs line, with low serum concentration and activin A induced definitive endoderm differentiation as detected by the expression of Sox17 and FoxA2. Moreover, addition of retinoic acid (RA), Noggin and Cyclopamine promoted pancreatic differentiation as indicated by the expression of the early pancreatic progenitor markers Isl1, Ngn3 and Pdx1. After maturation in suspension cultures with medium supplemented with fibronectin and insulin-transferring selenium (ITS), the differentiated cells expressed islet specific markers such as Pdx1, GLUT-2, Glucagon and Insulin. Finally, to complete the maturation process we added RSV to the suspension culture. The differentiated cells obtained by this approach comprised nearly 40% insulin-positive cells, almost 50% more than any other published differentiation protocol; contained numerous secretory granules and perfectly co-express Insulin and C-peptide, as detected by immunofluorescence. Furthermore, we demonstrated, using INS-1 cell line, that the effect of RSV was principally associated with a more efficient glucose-stimulated insulin secretion probably due to the activation of Sirt-1 and the subsequent inhibition of UCP2. So, as expected, differentiated hESCs treated with RSV showed an increase of Insulin and C-peptide secretion in response to glucose stimuli. In conclusion, our strategy allows the progressive differentiation of hESCs into pancreatic endoderm capable of generating mature β-cell-like cells and demonstrates that RSV could ameliorate the maturation process, delineating a possible missing step-forward necessary to obtain a more complete and efficient insulin-secreting cells differentiation strategy.

Poster Board Number: F-3084

DIRECTING CARDIOMYOGENIC DIFFERENTIATION OF HUMAN PLURIPOTENT STEM CELLS BY OVER EXPRESSION OF CARDIAC TRANSCRIPTION FACTORS - A NOTE OF CAUTION

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Purpose: Cardiomyocytes (CMs) derived from human pluripotent stem cells (hPSCs) possess a high potential for regenerative medicine. Previous publications suggested that a defined set of transcription factors (TFs) known to play pivotal roles in heart development also increased cardiomyogenesis *in vitro* upon (over)expression in mouse or human ES cells. To establish more efficient TF-combinations and to critically validate published data, hPSCs were transfected with expression vectors encoding for candidate factors and quantitative assessment of cardiomyocyte formation was performed. Methods: Using human cord blood-derived iPSC lines and hESC lines as well, expression plasmids were transfected by electroporation applying an efficient protocol recently developed by us. Differentiation was performed by adapting an established serum-free approach for cardiomyogenesis in hESC-embryoid bod-

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ies to monolayer differentiation, which was insufficient to induce cardiomyocytes when hPSC were transfected with control plasmids only. Anti- TroponinT, alpha-MHC, alpha-Actinin and Nkx2.5 -specific immunofluorescence staining was performed to quantify CM formation in response to the introduction of TFs. Results: In our approach Baf60c, Gata4 and Mesp1 (BGM) were found to be the most effective TF combination for hiPSC cardiomyogenesis . Removal of Baf60c slightly diminished CM formation while depletion of Gata4 or Mesp1 completely abolished CM formation. Each of these TFs alone did not induce CMs in our system. Although these results are encouraging we have notably observed sensitivity of the system regarding the cell density which depended on a number of factors including the seeding density and cell survival rates post-transfection. Conclusion: We have successfully established a non-viral, fast and straightforward system to test TF-induced cardiomyogenesis of hPSC and found a novel 3factor combination being most efficient. However, the overall efficiency of TF-induced hPSC transition into CMs was relatively low. Also, in line with previous reports based on monolayer or embryoid body differentiation, we found a critical impact of the (overall and local) cell density for cardiomyocyte specification *in vitro*. These results emphasize the necessity for detailed and cautious assessment when analysing effects of TF-directed lineage differentiation.

Poster Board Number: F-3085

CONTROLLED HOXB4 EXPRESSION DURING HESC HEMATOPOIETIC *IN VITRO* DIFFERENTIATION.

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hESC-derived hematopoietic cells represent a novel source for transplantable cells and a unique tool to examine early human development. It is well documented that hematopoietic differentiation can be enhanced by overexpression of HOXB4. HoxB4-expanded hematopoietic stem cells have also been shown to retain their normal potential for differentiation and long term self-renewal *in vivo* without the development of leukemia, suggesting that manipulation of HoxB4 expression might represent an effective way to expand functional hematopoietic cells for transplantation. However, it has been shown that enforced HoxB4 expression during hESCs hematopoietic differentiation resulted in a proliferative advantage in vitro, whereas neither improved blood colony formation nor engraftment was observed. At the same time it was shown that Lentiviral-Mediated HoxB4 expression in hESC initiates early hematopoiesis but does not promote myeloid differentiation. This led us to hypothesize that HOXB4 might modulate hematopoietic differentiation in time-dependent manner. To test our hypothesis, we developed a DOX-inducible HOXB4 expression system. hESCs line with inducible HOXB4 expression was generated and hematopoietic differentiation was induced. Using various timing of HOXB4 induction we demonstrated that hematopoietic differentiation was increased when HOXB4 was activated early in differentiating ESCs at the stage of hemangioblast prior to the emergence of hematopoietic cells. These data suggest that HoxB4-induced effects on hESC-derived hematopoietic cells are time-dependent during in vitro differentiation. Our study highlights the importance of HOXB4 expression and points to the need for experimental systems allowing controlled gene expression during differentiation.

Poster Board Number: F-3086

BMP4 PROMOTES HUMAN EMBRYONIC STEM CELLS TO UNDERGO EMT AND MESODERMAL COMMITMENT VIA SLUG AND MSX2

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Members of the Transforming Growth Factor-beta (TGF-beta) are essential regulators of embryonic development and disease, acting through serine/threonine receptors and intracellular Smad transcription factors. One member of the TGF-beta family, the Bone Morphogenetic Proteins (BMPs), has been shown to mediate differentiation in human embryonic stem cells (hES cells) but by what mechanism has not been clarified. The aim of the study was to dissect the role of the TGF-beta superfamily in hES cell early differentiation. The hES cells were stimulated with bFGF, and different members of the TGF-beta superfamily or adenovirally infected with different constitutively active TGF-beta superfamily receptor constructs. Diverse analytical methods were used, such as DNA microarrays, immunofluorescent stainings, western blotting, lentiviral shRNA infections, Chromatin Immunoprecipitations and migration assays. Our results demonstrate that BMP4 induces differentiation of hES cells by upregulating SLUG and MSX2, well-known transcription factors in Epithelial-Mesenchymal-Transition (EMT), and is a prerequisite process for gastrulation and tumour progression. BMP4 induced Smad1/5/8 phosphorylation co-localized with the SLUG protein on the edges of the colonies where hES cells undergo differentiation. The upregulation of the BMP targets is direct as shown by the binding of phosphorylated Smad1/5/8 to the SLUG and MSX2 promoter. Furthermore, using shRNA lentiviral vectors, we show that BMP4 induced MSX2 expression, leads to the differentiation of the mesodermal lineage. The results strongly suggest that BMP4 induces human ES cells to differentiate into mesodermal cells via the EMT mediators SLUG and MSX2. Our observations provide new insights into the mechanisms that regulate hES cell early commitment to the mesodermal lineage.

Poster Board Number: F-3087

FROM HESC TO MUSCLE, A PLATFORM FOR DEVELOPMENTAL RESEARCH AND THERAPEUTIC APPLICATION

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Human embryonic stem cell(hESC)is a good model for research of both embryonic development and regenerative medicine. Since the first hESC line established, many researches have proved that the pluripotent hESC can be directed into many kinds of functional adult cells in dishes. However, most of the reported methods induced differentiation through alteration of the growth factors in the culture medium. These methods are time-consuming, moreover, it is difficult to get the pure population of desired cells due to the low efficiency of induction. In this study, we use a lentiviral-based inducible gene expression system in hESCs to control the ectopic expression of MyoD, which is an essential transcriptional factor in skeletal muscle development. The induction of MyoD can efficiently direct the pluripotent hESCs into mesoderm in 24h,

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then go through the stage of proliferated myoblasts, finally form the multinucleated myotubes *in vitro*. The whole procedure took less than two weeks, with an induction efficiency over 90%. To our knowledge, it is the first time that hESCs being induced into terminal differentiated cells by only one factor. Also, this can be a potential resource for the cell therapy of the disease of muscle dysfunction in future.

Poster Board Number: F-3088

BMP4 SUFFICIENCY AND RESTRICTED NEUROEPITHELIAL CELL COMPETENCY FOR CHOROID PLEXUS EPITHELIAL CELL INDUCTION FROM MOUSE AND HUMAN EMBRYONIC STEM CELLS

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Choroid plexus epithelial cells (CPECs) secrete cerebrospinal fluid (CSF), establish the blood-CSF barrier, and play important roles in brain development and homeostasis. CPEC deficiencies have also been implicated in many neurologic and psychiatric disorders, which provides significant rationale for CPEC replacement and transplantation therapies. However, such applications are limited by the current inability to generate and expand CPECs in vitro. Here we show molecular, cellular, ultrastructural, and functional evidence for CPEC differentiation from mouse and human embryonic stem cell (ESC)-derived neuroepithelial cells (NECs). We first established an aggregation-based neural induction method, which showed highly efficient neural induction based on RT-qPCR and immunocytochemistry (ICC). Next, based on the known BMP signaling requirement for CPEC induction *in vivo*, we applied BMP4 to ESC-derived neural precursors at different time points. ESC-derived neural precursors displayed a restricted critical period for BMP4 responsiveness to induce CPEC gene expression, and this critical period correlated with the maximal induction of neuroepithelial cells (NECs) rather than radial glia (RG). BMP4 alone was sufficient to upregulate CPEC markers, at the expense of NEC markers, in a dose-dependent fashion. Further characterization of the "induced" CPECs (iCPECs) showed vesicular tissue formations and epithelial sheets that colabeled for the epithelial marker ZO1 and the CPEC specific markers Aqp1 and Ttr. By electron microscopy, the epithelial cells exhibited juxtalumenal tight junctions and abundant microvilli, as well as other ultrastructural features characteristic of CPECs, but not other neural cell types. Function of iCPECs was confirmed by their ability to self-assemble into vesicles on Matrigel, which grew and collapsed upon the addition of known CPEC secretion inhibitors, and AraC-enriched iCPECs were able to integrate into endogenous CPE with remarkably high efficiency after intraventricular injection. These data demonstrate that BMP4 is sufficient to induce CPEC fate in vitro and that ESC-derived NECs are selectively competent for CPEC fate, and pave the way for a CPEC-based regenerative medicine.

Poster Board Number: F-3089

MANIPULATION OF THE EXTRACELLULAR MICROENVIRONMENT BY MICRO- AND NANOTECHNOLOGY APPROACHES TO IMPROVE THE GENERATION OF PANCREATIC ENDOCRINE CELLS FROM HUMAN EMBRYONIC STEM CELLS

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Embryonic stem cells have the ability to generate all cell types in the body, and it is suggested that they may be able to provide an unlimited source for cell replacement therapy to treat degenerative diseases such as diabetes. The process of differentiating embryonic stem cells to functional insulin producing ß-cells *in vitro* is very complex, and extensive research into mechanisms driving the stepwise differentiation is required. The major focus has been on deciphering the role of soluble factors whereas the impact of the cell-matrix interaction has been mainly unattended. However, the chemical and physical properties of the natural extracellular matrix play a huge role in cellular behavior. Thus, understanding the role of the cell-matrix interaction is vital for the development of improved differentiation protocols. In this interdisciplinary study, a high throughput screening platform for surface designs has been developed and optimized where up to 500 different surface designs can be tested on a single microscope slide. With this high throughput screening platform it is possible to find surface designs which induce differentiation or proliferation within the various steps of human embryonic stem cells differentiation towards functional β-cells. The high throughput screen was tested in the first differentiation step, from human embryonic stem cells towards definitive endoderm, with combinations of various extracellular matrix peptides and proteins. Several surface combinations were found in the screen as possible inducers of definitive endoderm differentiation. Furthermore, this high throughput screening platform offers the opportunity to observe cellular behavior which is not seen in conventional cell culture format.

Poster Board Number: F-3090

REGULATION OF ACTIVIN/NODAL SIGNALLING DURING CELL-FATE SPECIFICATION IN THE MOUSE BLASTOCYST AND IN EMBRYONIC STEM CELLS

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Nodal, a secreted factor known for its conserved functions in cell-fate specification and the establishment of embryonic axes, is also required in mammals to maintain the pluripotency of the epiblast, the tissue that gives rise to all fetal lineages. Nodal expression begins at E3.5 in the inner cell mass (ICM) of the mouse blastocyst. When the ICM gives rise to the epiblast and the primitive endoderm (PrE) shortly before implantation, Nodal expression persists in both layers, albeit only transiently in the PrE. Although Activin/Nodal ligands of both embryonic and uterine origin appear to be

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broadly present at these stages, a sensitive fluorescent reporter transgene for an auto-regulatory (i.e. Activin/Nodal signalling dependent) Nodal enhancer, the ASE, exhibits heterogenous expression profiles in the ICM, the epiblast and the PrE. This suggested that not all cells in these tissues have the same ability to respond to Activin/Nodal signalling. We therefore investigated, both at the extra-cellular and at the intra-cellular levels, the mechanisms that modulate the transcriptional response to Activin/Nodal signalling. We derived ES cell lines from the transgenic ASE-YFP mouse line. We used inhibitor or enzyme treatments to disrupt major signalling pathways and glycosaminoglycans biosynthetic pathways in cultured embryos and in differentiating ES cells. Our results suggest a complex network of interactions, taking place both at the cell surface and within the cells, is involved in the regulation of Activin/Nodal signalling and modulates its participation in cell-fate decisions at preimplantation stages.

Poster Board Number: F-3091

SEX DEPENDENT GENE EXPRESSION IN EARLY HUMAN EMBRYONIC DEVELOPMENT

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Males and females demonstrate a large variety of sex dimorphic traits in spite of the relatively small difference in chromosomal content. These range from the evident anatomical and psychological differences to neuronal composition and disease susceptibility. It is widely accepted that most sexual dimorphic traits result from hormonal differences. However, differences between male and female embryos during early development, long before hormonal influence begins, suggest the presence of genetically driven dimorphisms. In this study we set out to identify Y chromosome related dimorphic phenotypes during early human development. To achieve this we compared the gene expression profiles of male and X-inactivated female human pluripotent stem cells. We discovered that the sex determining gene SRY is the only Y specific gene expressed in human male embryonic stem (ES) cells and reprogrammed induced pluripotent stem (iPS) cell lines. Using a reporter gene fused to the regulatory regions of SRY we found that both male and female pluripotent cells are able to recognize SRY promoter while somatic cells failed to do so. Furthermore iPS cells derived from XX-male sex reversion patients also show SRY expression indicating that SRY expression is activated in the pluripotent state. Y chromosome expression in males drives further dimorphic gene expression as expanding our analysis revealed more than 350 autosomal genes differentially expressed between male and female cells. Functional annotation analysis of the differentially expressed genes revealed involvement of developmental regulating and steroid metabolism pathways. The differential expression of ABCA1 and HSD17B12 in the steroid metabolism pathway was confirmed by qPCR and the functional relevance assessed by observing the cells response to estrogen precursor. Estrogen precursor (esteron) administration results in female ES cell proliferation while male ES cell growth is inhibited. Furthermore SRY expression in female ES cells resulted in growth inhibition after esteron administration and gene profile similar to male ES cells. Based on our result we propose that the presence of the Y chromosome and specifically SRY may result in difference in growth and differentiation properties between males and females.

Poster Board Number: F-3092

CONDITIONAL OVER-EXPRESSION OF MATH1 IN THE PRESENCE OF SPECIFIC EXTRINSIC FACTORS IN MOUSE EMBRYONIC STEM CELLS ORIENTATES NEURONS TOWARDS CEREBELLAR GRANULE CELL LINEAGE

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Development of the nervous system is a very balanced phenomenon directed by a vast array of intrinsic and extrinsic factors expressed in temporally and spatially restricted manner. Among the intrinsic factors, proneural genes coding for the basic helix-loop-helix (bHLH) class of transcription factors are both necessary and sufficient for the initiation of particular neural fate and to promote generation of progenitors committed to differentiate towards a particular lineage. Among them, Math1 has been shown indispensable for the generation of Cerebellar Granule Cells (CGC) and inner ear hair cells. CGC form around half of the total population of neurons in the brain and their loss is associated with many pathological conditions of the brain. Due to the limited regeneration capacity of adult nervous system, cell replacement therapy is seen as a promising method of curing such conditions. For the purpose, availability of homogeneous cultures with the desired cell population is critical. In the present study, mouse embryonic stem cells have been modified in a two-step process to generate a cell line with the inductive TET-ON system, in which Math1 is over-expressed only in the presence of Doxycycline (Dox). During the differentiation of the cell line towards neuronal lineage, embryoid bodies formation stage was chosen as the optimal stage for over-expression, which was also in line with the early expression of Math1 during CGC formation *in vivo*. Specific neuronal differentiation of the cell line was achieved in the presence of various growth factors documented to direct the generation of CGC and the efficiency of differentiation was analyzed at the end of various stages in non-induced control cultures (Ctl-f) or following Math1 over-expression (Dox-f). Math1 induction triggered an increase of its downstream target genes, including Mbh1 and Mbh2, and an increase in the expression of the early markers of the cerebellar territory (En1) and of cerebellar granule cell lineage (NeuroD1). Analysis of the cultures at the end of differentiation showed a marked increase in the CGC lineage markers GABAα6r and Zic1 in Dox-f cultures, although the total number of neural cells was found similar in both Ctl-f and Dox-f conditions. Conversely, other neuronal lineage markers like Serotonin and Tyrosine Hydroxylase were found significantly decreased in Dox-f differentiated cultures. Also, MyosinVIIa, a marker of inner ear hair cells, was found to be expressed similarly in both induced and non-induced condition. These results suggest that the combination of Math1 over-expression and specific inductive signals resulted in a specific enrichment of CGC. Thus as in *in vivo* conditions, a timely interplay of both intrinsic and extrinsic factor is important to specifically increase the population of CGC in vitro.

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Poster Board Number: F-3093

VESICLE TRANSFER BETWEEN ADULT CELLS AND MURINE PLURIPOTENT STEM CELLS

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Cell-secreted vesicles, including microvesicles derived from the plasma membrane and exosomes produced from multivesicular bodies, have been shown to play a role in intercellular communication. Importantly, recent studies have shown that both exosomes and microvesicles may contain mRNA and miRNA and can mediate transfer of their contents to distant cells. For cell repair or replacement therapies where cells require specific signals to direct their differentiation, the transfer of genetic information may be an ideal means of controlling that process. Pluripotent stem cells such as embryonic stem (ES) or induced pluripotent stem (iPS) cells are often studied as cell sources of choice for such therapeutic applications because of their ability to differentiate into all somatic cell types, but efficiently directing their differentiation is critical for their ultimate use and function. We hypothesized that facilitating genetic exchange between adult and pluripotent cells may provide a novel means of inducing directed differentiation for a specific application. In this study, vesicles were isolated from pre-osteoblasts using ultracentrifugation techniques and transferred to undifferentiated ES cell cultures. Vesicle populations were thoroughly characterized using several techniques, including transmission electron microscopy, Raman spectroscopy, differential light scattering, and flow cytometry; in parallel, cargo contained within the vesicles were identified by assessing the presence of protein and RNA. Incubation with vesicles stimulated cell proliferation and enhanced viability in the ES cell population. In separate studies, vesicles derived from both ES and iPS cells were examined and found to contain genetic information, which may contain potent regenerative cues and therefore be of significant use when transferred to diseased or injured cells. Horizontal gene transfer to or from pluripotent stem cells via vesicle distribution provides a novel method of manipulating recipient cell behavior through a concentrated and directed signal delivery. Future studies examining the incorporation of vesicles in a biomaterial delivery system may further facilitate the use of these naturally-derived genetic carriers.

Poster Board Number: F-3094

EFFECTS OF SACCHARIDES ON MOUSE ES CELLS -SURVIVAL, PLOLIFERATION AND DIFFERENTIATION

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In order to define the effects of saccharides on mouse embryonic stem cells (ES Cells), embryoid body (EB) formation and EB outgrowth, D-glucose of DMEM was substituted respectively with D-cellobiose, D-fructose, D-galactose, maltose monohydrate, D-raffinose, sucrose, trehalose or D-xylose, Glucose was also removed from serum. Glucose-deprived knockout-serum replacement (KSR) or glucose-deprived fetal bovine serum (FBS) was included in ES or EB medium. Proliferation and undifferentiated state of ES cells were tested in the control medium containing glucose and were also observed moderately in Xylose, Galactose, Fructose or Maltose-contained medium. EB's formation was poor in these saccharides, but their outgrowth in xylose and galactose was unique in their morphology and in the view of undifferentiated marker expression. Moreover, except for gene expression, the protein expression of undifferentiated markers such as Oct3/4 and Nanog had been keeping expressed in Xylose-cultured EB even though after culturing for a long time. It was speculated that xylose could have a novel function. Therefore, further experiments were performed focusing on Glucose and Xylose. The expression of undifferentiated markers such as Oct-3/4, Nanog and Rex-1 was kept in both monosaccharides for 8 days. High level expression of Oct 3/4 was disclosed either with real-time PCR or immunocytochemistry, but, LIF and feeder cells which are necessary to keep undifferentiation status of ES cells were used in glucose, not in xylose culture group. Futhermore, when co-culturing with PA6, though ES colonies differentiated into Tuj1-positive cells in Glucose, but they remained undifferentiated in Xylose. These results showed Xylose maintained the undifferentiated state in spite of ES or EB outgrowth. The application of Xylose in ES research is further explored.

Poster Board Number: F-3095

ADAPTATION OF A GLUTAMATERGIC *IN VITRO* DIFFERENTIATION PROTOCOL USING MURINE EMBRYONIC STEM CELLS DERIVED FROM HD MODELS

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In vitro differentiation of embryonic stem cells facilitates the generation of unlimited numbers of post-mitotic neurons as a tool for basic research, target identification, target validation, and drug discovery efforts in Huntington's Disease (HD). Based on a published protocol1, transgenic HD murine embryonic stem cells were directed towards CNS progenitors and glutamatergic neurons *in vitro*. In order to meet requirements for high content analyses, the protocol described here had been further optimized to enable a uniform distribution of neuronal cells for imaging. Characterization of *in vitro*-derived progenies for neuroepithelial, pan neuronal, glutamatergic, GABAergic and glia markers is routinely being performed on the OPERA system, a fully automated confocal microplate reader for high content imaging and high throughput screening. This technology offers image-based quantification to study phenotypic readouts of HD in mixed populations upon comprehensive time course analyses without the need to achieve essentially pure populations of neural cell types. In addition, it allows Calcium Flux analyses on individual cells upon glutamate induction as a proof that *in vitro*-derived cells become physiologically active. Overall, this stem cell based approach has been shown to fulfill main criteria for lineage-specific differentiation, such as efficiency, reproducibility and appropriate functional properties of differentiated cells, thereby providing a sophisticated *in vitro* model to study HD.References1Bibel et al. (2007) Generation of a defined and uniform population of CNS progenitors and neurons from mouse embryonic stem cells. Nature Protocols, Vol. 2(5), 1034-1043.

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Poster Board Number: F-3096

EXTRACELLULAR MATRIX PROTEINS ARE NECESSARY FOR MOUSE EMBRYONIC STEM CELL DIFFERENTIATION AND MAY GUIDE STEM CELL FATE

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While *in vitro* differentiation protocols often rely exclusively on soluble growth factors to direct mouse embryonic stem cell (mESC) fate, the ESC niche also contains fibrillar extracellular matrix (ECM) proteins, including fibronectin (FN) and laminin. Many soluble factors used in ESC differentiation are known to increase ECM expression, e.g. Activin A and FN, yet ECM's ability to direct ESC fate alone is not well understood and likely occurs at some points during development. To address this, we examined whether ECM proteins were necessary and/or sufficient to direct mESC differentiation into mesendodermal lineages. mESCs, grown for ten days as embryoid bodies under differentiating conditions in the absence of serum-form and cell-derived FN, maintained expression of the pluripotency marker, Nanog. The embryoid bodies also showed a spatiotemporal correlation between expression of FN and GATA4, a marker for differentiation, and an inverse correlation between FN and Nanog. When differentiated on gelatin, mESCs created a fibrillar ECM containing FN and laminin components-unlike conventional fibroblasts, which make FN only-while treatment with leukemia inhibitory factor inhibited fibrillar matrix production and maintained pluripotency. When endoderm growth factors Activin A and Wnt 3A are used in culture, cell-derived ECM composition changes, and this change is likely necessary but not sufficient to efficiently direct mESC differentiation. Together these data imply that FN is necessary for mESC differentiation and that the extracellular matrix may be an important director of stem cell fate.

Poster Board Number: F-3097

SCREENING OF LOW MOLECULAR COMPOUNDS THAT PROMOTE DIFFERENTIATION OF MOUSE ES CELLS TO PANCREATIC B CELLS

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The pancreatic transplantation and the pancreatic islet transplant are known aseffective treatments for type I diabetes. Donor shortage is an obstacle to the treatment of type I diabetes. The understanding of differentiation mechanism from ES cell to β-cells might add to our knowledge of the mechanism of β-cell differentiation and also contribute to the development of the regenerative medicine. The purpose of this research is to establish an efficient β-cell induction system using low molecular compounds. First, we aimed to establish a feeder free culture condition, in which ES cells can be induced into insulin producing cells *in vitro*. Feeder free system is useful to study the effect of low molecular compounds. We previously reported that mouse ES cell is efficiently differentiated into pancreatic progenitor cells and yielded as high as 30% Pdx1-positive cells, when they are cultured on M15 cells and added with growth factors (Shiraki et al., 2008). We also reported that M15 cell secretes extracellular matrices, which potentiate ES cells to differentiate into Pdx1-expressing pancreatic progenitor cells. The extracellular matrices can be reconstituted by a synthetic basement membrane (sBM) (Higuchi et al., 2010). Therefore, we speculate that culturing ES cells on a suitable scaffold would potentiate the differentiation into insulin producing cells. In an attempt for establishing a high throughput screening assay, we tested the use of three-dimensional scaffolds. Recently, we found that a novel synthetic matrix as a scafold can substitute sBM and ES cells cultured on this novel synthetic matrix differentiated into insulin producing cells. We found that the present culture system is feasible for screening for drugs that potentiate the differentiation of ES cells into insulin producing cells. After induction of pancreatic progenitor, we added chemical compounds into medium for screening. We have screened more than 1300 compounds and identified 10 compounds that could increase β-cell number. The chemical library which we used consisted of pharmacologically identified bioactive small chemical compounds. So we classified the hit compounds into several groups according to their target cascades. When using compounds that regulate different target pathways, we were able to achieve a higher effect by combining these compounds.

Poster Board Number: F-3098

REGULATION OF PDX1 GENE EXPRESSION BY NITRIC OXIDE TREATMENT ON DEFINITIVE ENDODERM CELLS DERIVED FROM MOUSE EMBRYONIC STEM CELLS.

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The transcription factor Pdx1 is required for the embryonic development of the pancreas and regulates the transcriptional network of pancreatic endocrine progenitor cells and ES cells-derived insulin producing cells. Thus, strategies addressed to the activation of Pdx1 and the pancreatic machinery using diverse molecules are important for differentiation and reprogramming protocols. We have studied how the exposure of mouse embryonic stem cells (mESCs) to 500μM of NO donor DETA-NO during 19h after 3 days of culture under LIF withdrawal, leads downregulation of Nanog and Oct4 and induce early differentiation events with acquisition of epithelial morphology and expression of markers of definitive endoderm, such as FoxA2, Gata4, Hnf1-β and Sox 17 and Pdx1. This phenotype was increased when cells were treated with valproic acid (VPA) for 10 days. In order to elucidate the mechanisms through which NO increase Pdx1 expression, we analyzed its regulatory region. There is not significant difference in global DNA methylation of distal and proximal CpG islands, although some unmethylated or methylated local CpG sites were found after treatment. Additionally, it was found the transcription factor Egr1 repressing the Pdx1 promoter, even though its inactivation do not cancel the total repressive action, suggesting additional mechanism such as interaction with some epigenetic complexes. Furthermore, acetylated H3 and H3K-4me3 were found on the Pdx1 proximal promoter. Based on these initial findings, we report the positive regulation of Pdx1 expression through the removal of Egr1, and activating post translational histone modifications in mES cells induced by treatment with NO. The interaction of Egr1 with other regulatory proteins is currently analyzed.

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Poster Board Number: F-3099

DYNAMIC HETEROGENEITY OF BRACHYURY EXPRESSION IN MOUSE EPIBLAST STEM CELLS

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Mouse Epiblast stem cells (EpiSCs) provide a platform for studying mammalian postimplantation development *in vitro*. EpiSCs is a heterogeneity and plasticity pluripotent populations, which is the most proximal cell line to the early somatic and germ precursors. However, it remains unclear whether the differention potential of subpopulation of EpiSC *in vitro* corresponding to distinct mouse pre-gastrulation developmental states. Here, we show that EpiSCs comprise two distinct subpopulations which distinguished by the expression of a specific Brachyury(T)-GFP marker, and T-GFP positive and negative EpiSCs are interchangeable. The two populations also show different developmental potential. The T-negative EpiSCs can efficiently undergo neuroectderm differention .T-positive EpiSCs resemble cells of pre-gastrulation mesendoderm. Our findings suggest that the heterogeneity of EpiSCs will guide cells differention into three germ layers immediately, corresponding to shortly gastrulation in mouse embryo.

Poster Board Number: F-3100

THE CD38/CADPR/CA2+ PATHWAY SUPPRESSES CARDIOMYOCYTE DIFFERENTIATION OF MOUSE EMBRYONIC STEM CELLS

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Intracellular Ca2+ mobilization modulates multiple cellular functions. Cyclic adenosine diphosphate-ribose (cADPR) is one of the endogenous Ca2+ mobilizers, which stimulates Ca2+ release in a wide variety of cell types. cADPR is formed by ADP-ribosyl cyclases from nicotinamide adenine dinucleotide (NAD). The main ADP-ribosyl cyclase is CD38, a multi-functional enzyme. To examine whether cADPR is involved in cardiac differentiation of mouse embryonic stem cells (mES), the expression of CD38 in mES cells was knocked-down by lentivirus-mediated short hairpin RNA (shRNA) targeting the mouse CD38 gene. We found that beating embryonic bodies (EBs) appeared earlier in CD38 knockdown or 8-Br-CADPR, a cADPR antagonist, treated cells than that in control cells. The percentage of beating EBs outgrows actually increased to 91% in CD38 knockdown cells compared to 43% in control cells. Real-time RT-PCR and western blot analyses showed that the expressions of several cardiac markers, includingGATA4, MEFsC, NKX2.5,a-MLC,were increased markedly in CD38 knockdown cells than those in control cells. Moreover, FACS analysis showed that more the cardiac Troponin T or a-actinin positive cardiomycytes existed in CD38 knockdown cells compared to that in control cells. Conversly, over-expression of CD38 in mouse ES cells significantly inhibits cardiomyocyte differentiation. Interestingly, three classes of typical action potential (APs): nodal-like, embryonic atrial-like, and embryonic ventricular-like, were all found in CD38 knock down EBs, and the percentage of each class had no significant difference compared to the control EB. Importantly, the CD38 knockdown ES-derived cardiomyocytes were similar to the wildtype ones in AP amplitude, APD50, APD90, Vmax, rate of APs, and response to modulating drugs, such as nifedipine and noradrenaline. In conclusion, our data indicate that the CD38/cADPR/Ca2+ signaling suppresses mES cardiac differentiation.

Poster Board Number: F-3101

HUMAN PLURIPOTENT STEM CELL DERIVED GABA NEURONS CORRECT LOCOMOTION DEFICITS IN A MOUSE MODEL OF HUNTINGTON DISEASE

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Degeneration of medium spiny GABA neurons in the basal ganglia underlies motor dysfunction of Huntington disease which lacks effective therapy. Cells therapy is a potential option for Huntington patients but it depends on the availability of functional medium spiny GABA neurons and their ability to reconnect the neural circuitry. By following the developmental principle, we have successfully directed human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) to enriched populations (87%) of DARPP32-expressing medium spiny GABA neurons. Transplantation of these human forebrain GABA neurons and their progenitors, but not GABA interneurons, into the striatum of quinolinic acid-lesioned mice results in generation of large populations of $DARPP32^+$ medium spiny GABA neurons. Importantly, these human medium spiny GABA neurons project specifically to the substantia nigra as well as receiving glutamatergic and dopaminergic inputs, thus reforming the cortex-striatum-midbrain circuitry. Finally, mice receiving the medium spiny GABA neurons but not the GABA interneurons exhibit recovery of motor deficits. This finding raises hopes for cell therapy for HD.

Poster Board Number: F-3102

DIRECT DIFFERENTIATION OF BRACHIAL LATERAL MOTOR COLUMNAR NEURONS FROM MOUSE EMBRYONIC STEM CELLS THROUGH HOXC6 OVER-EXPRESSION AND RA/SHH INDUCTION

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The widespread motoneuron death in neurodegenerative disease such as spinal muscular atrophy and amyotrophic lateral sclerosis leads to muscle denervation and denervation-induced muscle deterioration. Embryonic stem cell (ESC)-derived motor neurons could be an alternative used to restore motor function in these neurodegenerative diseases. Efficient locomotor control relies on precise connections between motor neuron subtypes and their target muscles. Therefore, acquisition of motor neuron subtype identities is a critical determinant to establish appropriate patterns of connectivity, since different neuron subtypes have different setting position, axonal trajectory and target choice. Previous studies have shown that ES cells could be induced into regional motor neurons with specific axonal trajectory and target choice in certain special condition. For example, the motor neurons generated from ESCs under RA/Shh conditions exhibit a rostral cervical, median motor column (MMC)-like identity. To generate motor neuron subtypes with brachial projection, we transfer Hoxc6 gene, a factor essential for development of brachial Lateral Motor Clolumnar neuron, into ES cells. In the expression construct, Hoxc6 was driven by 3.6kb HB9 enhancer and Hsp68 basal promoter. Then, the ES cells with Hoxc6

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were suspended in DFK10 medium for 2 days, followed by RA/Shh addition for another 5 days. The embryoid bodies (EB) were plated onto a cover slips and were cultured with the addition of GDNF (glial-derived neurotrophic factor),BDNF (brain-derived neurotrophic factor) and CNTF (ciliary neurotrophic factor) for 4 days. The cell types of the culture were examined by immuno-fluorescence test with a variety of antibodies, such as HB9, tubulin, Hoxc8. In our induction system, motor neurons could be generated efficiently, confirmed by HB9 and tubulin detection. When whole-cell patch-clamp recording techniques were applied, these motor neurons exhibit electrophysiological activity with repetitive trains of action potentials. Most importantly, some of the motor neurons clearly expressed Hoxc6 and Foxp1, which are the typical markers of brachial Lateral Motor Clolumnar neurons. Our results showed that forced expression of Hoxc6 in post-mitotic motor neurons was able to alter the Hox profile adequately and help some of the induced motor neurons to gain brachial Lateral Motor Clolumn (LMC) characters.

Poster Board Number: F-3103

THE ROLE OF HYPOXIA INDUCIBLE FACTOR-1A IN NEURAL COMMITMENT OF MOUSE EMBRYONIC STEM CELL

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Recent studies have shown that the floating aggregate culture of embryonic stem (ES) cells give rise efficient neural differentiation in growth factor-minimized medium. However, molecular mechanism that leads undifferentiated cells to neural fate in cell aggregates is not fully understood. Hear we demonstrate that the oxygen-sensitive hypoxia-inducible transcription factor, Hif-1α, is essential for neural commitment of ES cells. We found that hypoxic environment is spontaneously established in differentiating ES cell aggregates within three days, and that coincides with the Hif-1α activation. Adherent culture of ES cells under hypoxic condition resulted in Hif-1α activation and showed significant increase of the expression of neural progenitor specific-gene Sox1 compare to ES cells cultured under normoxic condition. Hif-1α-depleted ES cells showed severe reduction in Sox1 expression while still exhibiting high expression levels of the epiblast-marker gene Fgf5 by day 4, when the majority of the control ES cells had already shut off the expression. These findings indicate that Hif-1α is a positive regulator of neural conversion by promoting the transition of ES cell differentiation from epiblast state into neural progenitor state. We will discuss the mode of action of Hif-1α in early neural ectoderm differentiation.

Poster Board Number: F-3104

DYNAMIC CHANGES OF ERK SIGNALING ACTIVITY REGULATES MOUSE EMBRYONIC NEURAL STEM CELL PROLIFERATION, NEURONAL DIFFERENTIATION AND NEURITE OUTGROWTH

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Extra cellular signal-regulated kinase 1/2 (ERK 1/2) plays an important role in regulating embryonic neural stem cell (eNSC) fate. Here, we detected the activity of ERK1/2 signaling activity during brain development in mouse embryo. We found that during the eNSC expansion phase (embryonic day 9.5-10.5), ERK1/2 signaling was activated at a high level. However, low level of ERK1/2 activation was found during the neurogenic phase (E11.5-E13.5). After that phase, the ERK1/2 signaling was activated again at a high level. In order to further detect the relationship between the changes of ERK1/2 activity and the eNSC fate decision, we cultured the E10.5 eNSCs. A high level of ERK1/2 activity was observed in eNSCs cultured with the growth factors EGF and bFGF. After withdraw the growth factors, ERK1/2 signaling activity was decreased significantly and especially in the new generated neurons. Then a high level of ERK1/2 activity was detected in the neurite of the new neurons. Furthermore, we found that temporally inactivation of ERK1/2 activity in eNSCs by U0126 or a specific siRNA direct the eNSCs differentiated to neurons. However, sustain blocking the ERK1/2 activity inhibited the cell viability and decreased the neurite outgrowth of the new born neurons. Our results reveal the dynamic changes of ERK1/2 activity play a critical role in the embryonic neurogenesis.

Poster Board Number: F-3105

SYNERGISTIC ENHANCED DIFFERENTIATION OF MOUSE EMBRYONIC STEM CELLS TO HEPATOCYTES USING HYBRID EXTRACELLULAR MATRIX COMPOSED OF FUSION PROTEINS

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Hepatocytes are widely applied in the application of cell implantation, artificial liver or tissue engineering. However, the application was suppressed by the limitations in cellular proliferation and functional preservation, together with the difficulty in search for cell source. Thus the differentiation of embryonic stem (ES) cells to hepatocyte-like cells became important in providing a cell source for medical applications. A simple culture system to achieve the differentiated cell with high efficiency is crucial. In this study, undifferentiated ES cells cultured on E-cadherin-IgG Fc (E-cad-Fc) maintained high proliferative ability compared with the ones on gelatin-coated surface. Furthermore, the effect of hepatocyte growth factor - IgG Fc (HGF-Fc) on the differentiation of ES cells cultured on E-cad-Fc-coated polystyrene (PS) to hepatocyte-like cells was evaluated. HGF-Fc and E-cad-Fc were stably co-adsorbed onto PS surface without interference each other. Differentiation of ES cells to the hepatocyte-like cells was performed through four steps including mesendoderm, definitive endoderm, hepatic-progenitor cells and hepatocyte-like cells. The HGF-Fc in the E-cad-Fc / HGF-Fc hybrid extracellular matrix (ECM) did not affect the cellular morphology of single cells, the expression of E-cadherin. And the gene expression, albumin secretion of differentiated cells was confirmed in the hybrid culture system. The fusion protein of HGF-Fc cut down the requirements for the soluble factor of HGF in culture environment. After the hepatocyte induction and hepatocyte functions in the final stage of differentiation were confirmed, the induced cells were isolated by poly(*N-p*-vinylbenzyl-4-*O*-B-D-galactopyranosyl-D-gluconamide)(PVLA) matrix due to the specific interaction of asialoglycoprotein receptors and galactose in the PVLA. This hybrid ECM could be effectively used to enhance the hepatocyte differentiation, hepatocyte functions, and reduce the cost of induction process. It is promising in the application of mass production of ES cells-derived hepatocytes without enzymic stress and flow cytometry sorting process.

Poster Board Number: F-3106

THE EXPRESSION PROFILING AND FUNCTION OF MICRORNAS IN EARLY NEURAL DEVELOPMENT

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Neural fate commitment is a multi-step and complex event during early embryonic development. At early stage, the inner cell mass

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(ICM) differentiates to epiblast, and then the pluripotent epiblast cells give rise to all three germ layers including ectoderm. The process that ectoderm cells acquire a neural fate is called neural induction. Recently, we derive an in vitro epiblast stem cell (EpiSC) line from a critical time window during the neural differentiation of mouse ES cells. Thus mouse ES cells, ES cells derived EpiSCs (ESD-EpiSCs) and neural progenitor cells (NPCs) can be used as in vitro model to mimic the *in vivo* neural development. MicroRNAs are endogenous 21-23 nucleotide noncoding RNAs that can regulate almost all development processes. To test whether specific microRNAs are involved in different stages during the neural commitment, we used deep sequencing technology to perform microRNA expression profiling in ES cells, ESD-EpiSCs and NPCs. Through microRNA targets GO and BMP pathway components 3′-UTR luciferase assay we found that microRNAs are involved in early neural commitment, and mir-219 promotes neural differentiation of embryonic stem cells.

Poster Board Number: F-3107

THE EFFECT OF RECOMBINANT E-CADHERIN SUBSTRATUM ON DIFFERENTIATION OF MOUSE ES/IPS CELLS INTO PANCREATIC PROGENITORS

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Embryonic stem (ES) cells and induced pluripotent stem (iPS) cells provide unlimited cell source that can generate almost all cell types and treat degenerative diseases. Based on biomaterial approach to tissue engineering and regenerative medicine, recently we showed that using a fusion protein of E-cadherin extracellular domain and the IgG Fc domain (E-cad-Fc) as a model matrix for cell adhesion resulted in enhanced ES cells proliferation without colony formation. As for our previous study, this E-cadherin substratum was used to generate hepatocyte-like cells from mouse (m) ES cells. And it revealed that defined and selective culture conditions could overcome current problems such as chaotic differentiation and very low yield of mature cells. In this study, by applying E-cad-Fc matrix, we defined a new culture conditions for differentiation of mES/miPS cells into pancreatic progenitor cells. mES/miPS cells were cultured on E-cad-Fc in presence of Activin A to induce definitive endoderm. Then the cells were directed toward pancreatic progenitors in presence of well-defined protein kinase C (PKC) signaling activators including retinoic acid (RA), phorbol-12-myristate-13-acetate (PMA) and (-)-Indolactam V (ILV). Gelatin was used as a control extracellular matrix (ECM) for conventional culture system during the experiment. Immunostaining and RT-PCR were preformed to monitor the stage specific markers towards pancreatic progenitors. In contrast to gelatin, in the serum-free culture conditions and in the presence of significantly lower amount of Activin A, we could observe the efficient expression of definitive endoderm markers, Sox17 and Foxa2, and pancreatic progenitor markers, Pdx1 and Ptf1a. Therefore, the application of chimera protein-based recombinant ECM would be a step forward to develop a serum-/feeder-free defined culture system for homogeneous population of differentiated cells.

Poster Board Number: F-3108

ENHANCED CHONDROGENESIS BY SPECIFIC GROWTH FACTORS IN BUFFALO EMBRYONIC STEM CELLS MODEL

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The chondrogenic differentiation of embryonic stem cells (ESCs) via embryoid bodies has been established as a suitable model to understand chondrogenesis signaling pathway and molecular mechanism *in vitro*. This investigation was aimed to improve chondrogenic differentiation of buffalo ESCs by using bone morphogenetic protein 2 (BMP-2), fibroblast growth factor 10 (FGF-10), transforming growth factor-beta1 (TGF-β1) and their combinations. ESCs differentiation into chondrocytes was characterized by the appearance of alcian blue-stained areas as well as the expression of cartilage associated genes (RT-PCR) and protein (immunocytochemistry). BMP-2 or FGF-10 treatment separately enhanced chondrogenic differentiation, whereas TGF-β1 treatment inhibited buffalo ESC-derived chondrogenesis. Combined treatment of BMP-2 and FGF-10 mostly enhanced the buffalo ESCs chondrogenic differentiation showing 15.2 fold of alcian blue positive areas, 3.25 folds expression of the mesenchymal cell marker scleraxis gene, 1.9 and 7 folds expressions of the cartilage matrix protein collagen II gene and protein, respectively higher than untreated control groups. In addition, chondrogenesis to mature chondrocytes of the buffalo ESCs mesenchymal and chondrogenic progenitor cells was consistently recapitulated. This study suggests that the buffalo ESCs could be successfully triggered *in vitro* to differentiate into chondrocyte-like cells by specific growth factors. These findings may provide a novel *in vitro* model to study the regulatory mechanism of chondrogenesis.

Poster Board Number: F-3109

REGULATORY FACTORS FOR THE DIFFENTIATION OF CILIATED CELLS FROM MOUSE EMBRYONIC STEM CELLS

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Epithelial cells with motile cilia play crucial roles in the maintenance of homeostasis in adult organs and the morphogenesis during development. Defects in the structural components of cilia cause chronic bronchitis, sinusitis, situs inversus, male sterility and hydrocephalus. Most of the motile cilia have 9+2 microtubule arrangement with axonemal dyneins, which confer mechanical movement to cilia. These 9+2 cilia can be observed in the respiratory tract, reproductive organs, and brain ependyma. In contrast, primary cilia, which have 9+0 skeletal structure, are basically immotile because they lack axonemal dynein. Primary cilia exist singly in almost all cells, and serve as sensory organelles for various extra cellular stimuli. As one of the exception of this rule, there are 9+0 cilia in

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the embryonic node that move and generate extra-embryonic fuid fow. Mammalian ciliogenesis consists of four stages: (1) duplication of centrioles, (2) migration and docking of centrioles to the apical membrane, (3) formation of accessory structures of basal bodies and (4) elongation of cilia. Although the morphological studies by electron microscopy have uncovered detail of these processes, regulatory factors involved in ciliogenesis during development are largely unknown. Foxa2 is a Forkhead box (F-box) transcription factor is a crucial transcription factor that direct specification of the definitive endoderm formation such as respiratory tract, liver, pancreas and intestine. In the lung, the expression of Foxa2 is restricted to the epithelial cells of conducting and peripheral airways and alveoli. Previous studies revealed that Foxa2 enhanced the expressions of respiratory epithelial marker gene, In this paper, we have shown that Foxa2 and Foxj1 are crucial regulatory molecules for the ciliated cell differentiation from ES cells. Ectopic expression of Foxa2 in mouse ES cells induced trachea-type ciliated cells. In contrast, ectopic expression of Foxj1 induced ependymal cell-like ciliated cells from ES cells, although Foxj1 has been reported as an essential transcription factor for ciliated cell differentiation. Our results suggest that transcription factors regulates the type of ciliated cell differentiation and that Foxj1, the essential factor for the ciliated cells, is not sufficient to control the differentiation of ciliated cells from ES cells.

Poster Board Number: F-3110

EXPRESSION OF TEKT1 DURING DIFFERENTIATION OF MONKEY ES AND IPS CELLS

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Primate embryonic stem (ES) cells reportedly expressed post-meiotic marker genes such as TEKT1 and GDF9, specific for spermatid and oocyte respectively, during spontaneous differentiation *in vitro*. Expression of TEKT1 and GDF9 has also been reported regardless of sex difference (XY or XX) of primate ES cells. In this study we focused on the expression of TEKT1 as well as other germ cell markers during *in vitro* differentiation using XY and XX ES and XX iPS cell lines established from cynomolgus monkey. Immunohistochemical analysis was also performed to identify cells expressing these markers in EBs. In addition, we investigated the cells expressing TEKT1 using TEKT1 reporter ES cell line. Pre-meiotic germ cell markers (VASA, NANOS3, PIWIL2) were upregulated in both ES and iPS cell lines upon the *in vitro* differentiation. Up-regulation of post-meiotic markers (HAPRIN, TEKT1, and TEKT3) was observed in both ES and iPS cell lines. Of interest, ES and iPS cells showed similar time course of germ cell marker genes. These results suggested that non-human primate iPS cells also express post-meiotic markers and sex difference (XY or XX) did not affect the expression pattern of TEKT1 and under *in vitro* differentiation. Immunohistochemical analyses of EBs showed specific localization of cells expressing TEKT1 protein. We are currently analyzing gene expression profiles of TEKT1 expressing cells and will discuss its character.

Poster Board Number: F-3111

DIFFERENTIATION OF MOUSE EMBRYONIC STEM CELLS INTO INNER EAR HAIR CELL-LIKE CELLS USING STROMAL CELLS CONDITIONED MEDIUM

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[Introduction] Hearing loss is mainly due to an inability of cochlear sensory epithelia to replace lost hair cells of the inner ear. Embryonic stem (ES) cells have shown promise for cell therapy in a range of organs, because of their potential for self-renewal and pluripotency. In the present study, we investigated the differentiation of ES cells into inner ear hair cells using differentiation-inducing activity in culture supernatants of PA6 and ST2 stromal cells. [Methods] Various media, including DMEM with 10% FBS, DMEM+KOS, DMEM+N2M, DMEM+N2M+FGF2, PA6-conditioned medium (PA6-CM), and ST2-CM, were investigated in regard to their effects on the differentiation of 4-day embryoid bodies (EBs) into hair cell-like cells. EBs were cultured in media for 14 days, after which the expressions of hair cell markers, including myosin 7a, myosin 6, Brn3c, and a9AchR, were examined by RT-PCR and immunocytochemistry. [Results] Prominent neuritic outgrowths were observed emerging from the EB aggregates in cultures with ST2-CM, while such distinguished formation of neuritis was not observed in cultures with the other media. PA6-CM induced expressions of MAP2 and GFAP, but not those of inner ear hair cell markers, while it promoted the induction of hair cell markers, as shown by both RT-PCR and immunocytochemistry findings. [Conclusion] Inner ear hair cell marker-positive cells were generated from ES cells after culturing in ST2-CM, suggesting that secreted differentiation-inducing activity may be sufficient to induce hair cell-like cells from ES cells.

Poster Board Number: F-3112

ROBUST AND SCALABLE GENERATION OF PROGENITOR T CELLS FROM HEMATOPOIETIC STEM CELLS (HSCS) AND PLURIPOTENT STEM CELLS (PSCS) IN A NOTCH LIGAND-FUNCTIONALIZED HYDROGEL SYSTEM

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Embryonic and induced PSCs hold great potential for cell-based therapies due to their ability to differentiate into all cell types, including T cells. T cells are critical for regulating immune responses and maintaining an immunological memory of recurring pathogens in the body. T cell deficiency post chemotherapy can be lethal as these cells mediate resistance to opportunistic infections after bone marrow transplantation. Engineering robust technologies to generate progenitor T cells from HSCs and PSCs could provide a scalable source of transplantable immune cells and important tools for modeling immune diseases. T cells develop in the thymus in a sequential, temporal and spatially ordered microenvironment. To engineer the thymic niche *in vitro*, a novel immobilized ligand-hydrogel system is being developed as a defined synthetic stem cell niche for T cell progenitor development and expansion. Our laboratory has previously shown the efficacy of using agarose hydrogels functionalized with growth factors to encapsulate and differentiate PSCs towards the mesoderm lineage. Here, we present work functionalizing agarose with protein-G that is capable of immobilizing two Notch signaling ligands essential for T cell development - Delta-like 4 (DL4) and Jagged-1 (Jag-1). The initial

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immobilization chemistry was optimized using IgG-FITC, a model protein for the Fc-expressing Notch ligands. We obtained covalently-immobilized IgG-FITC at a concentration of 40ug/mL while the non-specifically adsorbed control had a concentration of 6.7ug/mL. Additionally, to optimize the three-dimensional presentation of Notch ligand, we coupled IgG-FITC agarose with the Aggrewell system for the microwell-based aggregation of defined HSC numbers in functionalized agarose. Results will be presented quantifying and functionally validating the conjugation of DL4 and Jag-1 to agarose using Notch-responsive model cell lines. Optimal local cell density and Notch ligand ratios will be determined using mouse fetal liver-derived HSCs. We have previously presented the differentiation kinetics of mouse ESCs and iPSCs derived from mature and immature T cell subsets (T-iPSCs) to blood progenitor cells in serum-free conditions with defined aggregate sizes. We observed that double-negative 1 (DN1) progenitor T cell derived iPSCs (DN1-iPSCs) produced six-fold more Flk1+PDGFRa+ blood progenitors than ESC controls as early as day 3.5 of serum-free differentiation. These T-iPSC derived mesoderm progenitors will be differentiated back to progenitor T cells in the DL4/Jag-1 functionalized agarose system. The assay will be used to validate whether differentiation of T cells from stem cells can be accelerated by deriving iPSCs from mature or immature T cell subsets (or T-iPSCs) as they retain memory of the cell type from which they are derived. By engineering an immobilized Notch ligand-hydrogel system as a defined synthetic stem cell niche, we aim to produce progenitor T cells with high yield and efficiency and gain greater insight into the mechanisms that drive PSCs to the definitive mesoderm fate.

Poster Board Number: F-3113

ELL3 ENHANCES DIFFERENTIATION OF MOUSE ESCS BY REGULATING EMT AND APOPTOSIS

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Ell3 is a testis-specific RNA polymerase II elongation factor whose cellular function is not clear. The present study shows that *Ell3* is activated during the differentiation of mouse embryonic stem cells (mESCs) where it plays a critical role to stimulate lineage differentiation of mouse embryonic stem cells (mESCs) by promoting epithelial-mesenchymal transition (EMT) and suppressing apoptosis. mESCs engineered to stably expressing *Ell3* were rapidly differentiated compared to control cells either under spontaneous differentiation condition or neural lineage specific differentiation condition. Gene expression profile and quantitative RT-PCR analysis showed that the expression of EMT markers including Zeb1 and Zeb2, two major genes to regulate EMT, were activated in *Ell3* overexpressing mESCs. Remarkably, knockdown of Zeb1 compromised the enhanced differentiation capacity of *Ell3* overexpressing mESCs, which indicates that *Ell3* plays to induce differentiation of mESCs by activating EMT. In contrast to *Ell3* overexpressing mESCs, *Ell3* knocked-down mESCs could not differentiate in the differentiation condition but instead underwent caspase-dependent apoptosis. In addition, apoptosis of differentiating *Ell3* knocked-down mESCs was associated with the enhanced expression of p53. The present results suggest that *Ell3* promotes the differentiation of mESCs by activating the expression of EMT related genes and by suppressing p53 expression.

Poster Board Number: F-3114

CHARACTERIZATION OF MESP1 POSITIVE CARDIOVASCULAR PROGENITORS DERIVED FROM EMBRYONIC STEM CELLS

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Embryonic stem cells (ESCs) are a potent tool to study cardiovascular progenitor development in vitro. Recent ESC studies suggested that Flk1+/PDGFRa+ cells represent an early cardiovascular progenitor population that give rise to heart and vascular cells, but it is unknown if these cells co-express the earliest cardiovascular progenitor marker Mesp1, whose lineage contributes to nearly all heart and vessel cells *in vivo*. To address this question, we have generated a Mesp1RFP ESC line that constitutively express RFP in cells derived from Mesp1+ progenitor. In this line, Cre is expressed from the Mesp1 locus and activate RFP expression by excising a loxP-stop-loxP sequence in the Rosa locus. With an established cardiomyocyte differentiation protocol, we induced Flk1+/PDGFRa+ cells using the Mesp1RFP ESCs. On day 4 of ESC differentiation, approximately 50% of ESCs were differentiated to Flk1+/PDGFRa+ cells. Surprisingly, only a small fraction of Flk1+/PDGFRa+ cells expressed RFP. We further analyzed the RFP+ cells by flow cytometry and found that RFP+ cells can give rise to cardiac cells more efficiently than Flk1+/PDGFRa+ cells. These results suggest that a subset of Flk1+/PDGFRa+ may represent "pure" cardiovascular progenitors in the ESC system. Further investigation on Mesp1+ cells will provide fundamental insights into understanding the induction and differentiation of cardiovascular progenitors.

Poster Board Number: F-3115

CARDIAC DIFFERENTIATION OF MOUSE EMBRYONIC STEM CELLS BY PATTERNING CULTURE METHOD

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Heart transplantation is limited by the shortage of donor hearts. Cardiac regenerative therapy based on cardiomyocyte transplantation is expected to become an alternative to heart transplantation. Investigations aiming to prepare cardiomyocytes for transplantation from embryonic stem cells and induced pluripotent cells (ES/iPS cells) are actively conducted all over the world. A human left ventricle contains several billions of cardiomyocytes, and it is estimated that about a billion cardiomyocytes are lost by myocardial infarction that induces heart failure. This fact suggests that preferably more than a billion cardiomyocytes are necessary to restore severe heart failure. To prepare such a large amount of cardiomyocytes from ES/iPS cells practically, cell culture methods must be improved to reduce requisite labor and costs as much as possible. There are two conventional culture methods for cardiac differentiation of ES/iPS cells, a hanging drop method and a suspension culture method. A hanging drop method can control the size of embryoid bodies (EBs) for efficient cardiac differentiation. However, the drawback is that it requires labor-intensive procedures and is thus unfavorable for large-scale culture. On the other hand, a suspension culture method is favorable for large-scale culture. However, the drawback is that it cannot control the size of EBs, so

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that the efficiency of cardiac differentiation is relatively low. To overcome these drawbacks, in the present study, we developed a novel culture method "patterning culture method". This method enables simple mass preparation of size- and shape-controlled cell aggregates for efficient cardiac differentiation on culture substrates. Utilizing photolithography, polyacrylamide micro-patterns were grafted onto the silanized surfaces of glass coverslips. The silanized glass surfaces are cell-adhesive and the polyacrylamide-grafted surfaces are non-cell-adhesive because of its high hydrophilicity. Therefore, the surface of this substrate comprised a micro-pattern of cell-adhesive and non-cell-adhesive domains. Mouse ES cells, in which α-cardiac myosin heavy chain promoter-driven EGFP gene was introduced, were cultured on this cell patterning substrate. As the ES cells proliferated, they spontaneously formed cell aggregates on the cell-adhesive domains, and the aggregate-induced cardiac differentiation was confirmed by beating and EGFP expression. Morphological analysis revealed that the formation of ES cell aggregates resulted from the over-proliferation of cells at the edges of cell-adhesive domains due to the failure of contact inhibition. The shape and size of ES cell aggregates were controlled by the configuration of micro-pattern on the substrate, and the efficiency of cardiac differentiation also changed depending on that configuration. The investigation is ongoing to clarify the optimal configuration of micro-pattern for the efficient preparation of cardiomyocytes from ES/iPS cells.

Poster Board Number: F-3116

PLURIPOTENCY FACTORS GOVERN MESENDODERM FORMATION

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Pluripotency factors have been shown to regulate ES cell self-renewal. However, their function in early embryonic lineage commitment is poorly understood. In this study we identify a novel pluripotency gene in the onset of mesendoderm specification. We show that this gene is dynamically upregulated in mouse embryos and *in vitro* differentiated embryonic stem cells, which is concomitant with mesendodermal lineage specification. Genome-wide transcriptional profiling of *in vitro* differentiated murine embryonic stem cells indicated an enhanced specification towards mesendoderm. Accordingly, prolonged cultures were enriched for mature mesodermal and endodermal derivatives. We demonstrate that this novel factor directly activates key transcription factors of mesendoderm as assessed by ChIP and luciferase assays. In addition, there is a paracrine mechanism governed by this gene, which in turn activates the non-cell autonomous cell fate specification. Taken together, our data suggest a cell autonomous and non-cell autonomous function of a so far unknown pluripotency factor in early embryonic lineage specification towards mesendoderm.

Poster Board Number: F-3117

S14 INDUCES NEUROGENESIS IN RAT FETAL NEURAL STEM CELLS

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Neural stem cells (NSC) have ability to proliferate and differentiate into cells that compose the nervous system such as neurons and glia. In our current study, we screened several components that are isolated from natural products and found a compound (S14) that was effective in induction of neuronal differentiation in NSC derived from fetal rat cortex. Neurogenic effect of S14 was determined by immunocytochemistry, real-time RT-PCR, and western blot analysis. S14 increased the number of neurons in a concentration-dependent manner (0.25-1uM) and induced genes related to neurogenesis such as *βIII-tubulin*. S14 also increased Tuj1 protein level in a concentration-dependent manner. However, S14 had no effect on NSCs proliferation. Cell proliferation was assessed by measuring neurosphere size and by MTT assay. The sphere volume of S14-treated NSCs was not increased compared with that of control. Cell viability showed no significant changes. S14 also decreased the expression of stem cell marker *nestin* mRNA and nestin protein (western blot analysis). Our results suggest that natural product can regulate NSC fate and may be useful for future regenerative medicine.

Poster Board Number: F-3118

EXPRESSION OF CONNEXINS IS REQUIRED FOR MESO- AND ENDODERMAL DIFFERENTIATION OF ES CELLS *IN VITRO*.

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Gap junctions are intercellular conduits that allow the diffusional exchange of small molecules (< 1.8 kDa) between neighboring cells. A gap junction channel consists of two hemichannels (connexons), hexameric structures formed by protein subunits, termed connexins. 20 connexin isoforms have been identified in the mouse, 21 in the human genome. Mouse embryonic stem (ES) cells express three connexin isoforms (Cx31, Cx43 and Cx45) and are functionally coupled via gap junction channels. The actual role of these connexins in terms of pluripotency maintenance and differentiation remains elusive. To elucidate the function of gap junctional communication we generated loxP-modified ES cells for the conditional ablation of both, Cx43 and Cx45. Cx43/Cx45-deficient cells show 95% reduction in gap junctional intercellular communication (GJIC) as determined by neurobiotin microinjection. Remarkably, this strong reduction in cell-cell coupling does not alter proliferation rates or apoptotic cell death. Differentiation of Cx43/Cx45-deficient ES cells employing an embryoid body (EB) differentiation paradigm revealed no obvious morphological differences to the loxP-modified control cells until day five. However, whereas between day six and eight Cx43/45 loxP-modified EBs undergo strong morphological changes, including the development of cystic structures, connexin-deficient EBs do not change. Even after 17 days in suspension culture only a few cystic EBs were detected in the Cx-null cultures. Moreover, in the mutants we found a strong reduction in the number of EBs showing beating clusters and in the expression of various cardiac proteins. Next we addressed the question whether the expression of mRNAs associated with early ES cell differentiation are misregulated upon Cx43/Cx45 deficiency. Therefore, we performed quantitative RT-PCR analyses to determine the expression levels of early mesoderm and endoderm markers. We found that the expression of these mRNAs was significantly reduced in Cx43/Cx45 deficient EBs at several time points. This phenotype was rescued by the lentiviral overexpression of Cx43 and Cx45 in Cx43/Cx45 deficient EBs. From these observa-

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tions we conclude that connexin expression is required for the proper differentiation of ES cells in suspension culture. We assume that connexin expression is at least essential for the induction of primitive streak-like structures and mesendodermal differentiation *in vitro*.

Poster Board Number: F-3119

EFFICIENT HEMATOPOIETIC DIFFERENTIATION OF COMMON MARMOSET EMBRYONIC STEM CELLS BY THE INHIBITION OF PI3K-AKT PATHWAY

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The human regenerative medicine by the transplantation of the functional cells differentiated from embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) have great potential of contributing to the treatment for various diseases, and thus have attracted huge public attention. However, the risk of unwelcome tumor formation originated from transplanted cells in recipients remains to be solved. Therefore the safety and efficacy of ESCs/iPSCs based therapies should be carefully examined using reliable animal models for diseases before their clinical application. Among experimental animal models, common marmoset (CM, Callithrix jacchus), one of NEW WORLD monkeys, has recently been recognized as a useful non human primate because they are small, easy to handle, highly reproductive and genetically very similar to humans. We have continuously investigated the characteristics of ESCs and iPSCs derived from CM. It has been reported that the maintenance of human or mouse ESCs/iPSCs require basic fibroblast growth factor (bFGF) or leukemia Inhibitory factor (LIF) respectively, however the growth factors required for the culture of CM ESCs/iPSCs have not been clearly determined. To clarify whether self-renewal and proliferation of CM ESCs depend on bFGF or LIF, we cultured them in various conditions, and found that the undifferentiated state of CM ESCs could be maintained in the medium (Knockout DMEM supplemented with 20% Knockout Serum Replacement, 1 mM L-glutamine, 0.1 mM MEM nonessential amino acids, 0.1 mM β-mercaptoethanol and antibiotics) containing bFGF even when they were passaged by mechanically dissociated single cells. Moreover the proliferation of CM ESCs was significantly enhanced in the presence of bFGF. Addition of LIF in the culture medium did not affect the self renewal and proliferation of CM ESCs. These results indicate that bFGF is necessary for culturing CM ESCs, but LIF is dispensable. The bFGF is known to activate PI3K-AKT pathway to maintain them in an undifferentiated state of human ESCs. To develop an efficient method to promote differentiation of CM ESCs into hematopoietic cells by the inhibition of PI3K-AKT pathway, we treated two CM ESC lines (Cj11 and CM40) with PI3K inhibitor, LY294002, during embryoid body (EB) formation for 4days. We found that addition of LY294002 increased the expression of mesoderm (T and FLK1), hematopoietic and endothelial (CD34) makers in Day4-EBs, although the number of EBs was significantly decreased. Moreover colony formation assay revealed that the ability of hematopoietic potential of Day4-EBs was also enhanced in the presence of LY294002. These results suggested that inhibition of PI3K pathway promoted the hematopoietic differentiation of CM ESCs during EB formation. Our findings provide useful information to develop a better technology of the culture and hematopoietic differentiation of CM ESCs.

Poster Board Number: F-3120

EFFICIENT DERIVATION OF NEURAL STEM CELLS FROM COMMON MARMOSET ES CELLS AND IPS CELLS.

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The Common marmoset (Callithrix jacchus) is a small New World primate spiecies. They generate many offsprings during their life, and are easy to breed in a laboratory, compared to other types of primate models. Thus, they have been expected as non-human primate models for varieties of purposes including pre-clinical studies. We have previously found that the transplantation of neural stem cells (NSCs) derived from mouse and human ES cells and iPS cells are effective for functional recovery of mouse spinal cord injury models (Kuamagai et al., 2009, Tsuji and Miura et al., 2010, Nori and Okada et al., 2011). However, for clinical application, these transplantation studies, using rodent models or xenograft transplantation of human cells into rodent models, are insufficient to evaluate immunological rejections or tumorigenesis of the transplanted NSCs. Thus, allograft transplantation in primate models, such as transplantation of marmoset NSCs into marmoset spinal cord injury model, is essential. We have also previously established marmoset ES cells (Sasaki et al., 2005) and marmoset iPS cells (Tomioka et al., 2010). In the present study, by modifying our own methods for neural differentiation from mouse or human ES and iPS cells (Okada et al., 2008, Okada et al., manuscript in preparation), we derived NSCs from marmoset ES cells efficiently, which are applicable to the transplantation into spinal cord injury model or the study of neural development of primates. We first formed embryoid bodies (EBs) from marmoset ES cells in the presence of neural inducers, Dorsomorphin and Retinoic acid, and then formed neurospheres by dissociating EBs and culturing them in suspension in the presence of FGF-2. As a result, the treatment of Dorsomorphin and Retinoic acid during EB formation significantly increased neurosphere formation efficiency. Marmoset ES cell-derived neurospheres could be passaged repeatedly, and could give rise to neurons and astrocytes when cultured adherently without FGF-2. Importantly, neurons derived from these neurospheres showed significant Ca2+ increase on high K+ stimulation, suggesting that these neurons may be functional. Moreover, by modifying this method, we could obtain neurospheres which could generate oligodendrocytes. In the near future, by the transplantation of marmoset ES cell and iPS cell-derived neurospheres into marmoset spinal cord injury models, we are going to examine not only functional recoveries, but also the effectiveness of NSCs transplantation in the aspect of immunological rejections and tumorigenicities in primate allograft models.

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Poster Board Number: F-3121

CELL FATE SPECIFICATION IN EMBRYONIC STEM CELL DERIVED MOTOR NEURONS: DECIPHERING THE TRANSCRIPTIONAL CODE OF PHRENIC MOTOR NEURONS.

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All land vertebrates use lungs to breathe air. Inspiration and expiration are driven by complex brainstem circuitry, which relays the breathing rhythm via a motor neuron (MN) output layer to respiratory muscles. While considerable progress has been made in understanding the central pattern generator itself, very little is known about how respiratory MNs form and connect to target muscle during embryogenesis. We have established a gain-of-function (GOF) assay based on the differentiation of mouse embryonic stem cells (ESCs) to MNs that allows us to systematically screen for genes involved in MN subtype specification. We applied this approach to the developmental program of phrenic neurons, a key population of respiratory MNs that connects to the diaphragm muscle. We initially identified a set of genes enriched in the phrenic nucleus by comparing expression profiles of three primary MN populations isolated from E11.5 mouse embryos. Phrenic MNs, non-phrenic MNs from the same mid-cervical level and limb-innervating MNs were sorted by FACS and analyzed with Affymetrix arrays. The transcription factors up-regulated in embryonic phrenic MNs were then assessed for their ability to impose a (partial) phrenic identity in vitro. To this end, we generated a transgenic mouse ESC line expressing the CD2 surface marker fused to GFP under the control of the MN-specific Hb9 promoter. The ESCs also carry a doxycycline-inducible promoter followed by a loxP site, which allowed us to insert candidate genes into this locus by Cre-mediated recombination. In vitro differentiation of these ESCs generated 20-30% MNs that were then magnetically enriched and induced with doxycycline for 30h in culture. The acquisition of phrenic-like transcriptional profiles in response to candidate gene expression was evaluated using the data from primary phrenic MNs and control populations. We studied the effect of 15 transcription factors with this system and identified several potential determinants of phrenic identity, including Pou3f1. For example, ectopic expression of Pou3f1 in ESC-MNs up-regulated all type-II cadherins and protocadherins previously detected in primary phrenic neurons. Since phrenic identity is likely to be established by a combinatorial code of multiple factors, our future work will focus on other determinants that interact with Pou3f1. In addition to the in silico analysis, we are testing the ability of candidate factors to impose phrenic-like axonal projections *in vivo*. To expose ESC-MNs to a normal embryonic environment, we grafted them into cervical segments of intact mouse embryos in whole embryo cultures. The outgrowing motor axons follow normal trajectories, and the ability of Pou3f1 and/or other candidates to redirect them into the phrenic nerve is currently being investigated. GOF screens with tissue derived from ESCs, such as the one with MNs reported here, is a powerful tool to study developmental programs, particularly when the use of large numbers of genetically modified mice is not practical. Furthermore, we intend to apply the insights gained into neuronal subtype specification to human ESC-MNs. Artificial human neural tissue could be developed as a tool for cell replacement therapy for conditions in which neurons are lost due to degeneration or injury. The success of such a therapeutic strategy may rely on the imposition of the precise neuronal subtype required in the graft.

Poster Board Number: F-3122

TAP63, MEMBER OF THE P53 FAMILY, IS ESSENTIAL FOR HEART DEVELOPMENT AND CARDIOGENESIS OF EMBRYONIC STEM CELLS

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p63, a member of the p53 family, is essential for skin morphogenesis and epithelial stem cell maintenance. p63 gene encodes for two main isoforms, TAp63 and DNp63 with distinct gene expression profiling and opposite functions. While TAp63 controls cell apoptosis and ageing, DNp63 is implicated in cell proliferation, cell adhesion and epidermal commitment. Here, we report an unexpected role of TAp63 in cardiogenesis. p63 null mice exhibit severe defects in embryonic cardiac development, including dilation of both ventricles, a defect in trabeculation and abnormal septation. This was accompanied by myofibrillar disarray, mitochondrial disorganization and reduction in spontaneous calcium spikes. p63-deficient embryos have a decreased pool of nkx2.5, tbx5 and isl1-positive progenitor cells, suggesting that p63 is required at early stage for proper heart development from both the first and second heart lineages. By the use of embryonic stem (ES) cells, we show that TAp63 deficiency prevents expression of pivotal cardiac genes and production of cardiomyocytes. TAp63 is expressed *in vivo* and in vitro by endodermal cells. Coculture of p63-knockdown ES cells with wild type ES cells, supplementation with Activin A or over-expression of GATA-6 rescue cardiogenesis. Therefore, TAp63 acts in a non-cell-autonomous manner by modulating expression of endodermal factors. Our findings uncover a critical role for p63 in cardiogenesis that could be related to human heart disease.

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GENERATION OF DOPAMINE-SECRETING CELLS FROM NURR1 EXPRESSING ES CELLS CULTURED ON ELECTROSPUN POLY EPSILON CAPROLACTONE GELATIN NANOFIBROUS SCAFFOLDS

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Parkinson's disease (PD) is a neurodegenerative disorder in which the nigro-striatal dopaminergic neurons selectively have been lost. Cell replacement therapy for PD has received a great deal of attention in the last decade. Many investigations have used pluripotent stem cells including embryonic stem (ES) and induced pluripotent stem (iPS) cells for generation of dopaminergic (DAergic) neurons. For transplantation of ES-derived DAergic neurons to PD subjects, a main issue is to culture and differentiate ES cells on substrates that can firstly provide a niche for efficient DAergic differentiation, and secondly facilitate the transferring and implantation of ES-derived DAergic neurons to midbrain of PD mice model. The present study

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was aimed to study the effect of three dimensional cell culture provided by electrospun poly(epsilon-caprolactone)/gelatin (PCL/Gelatin) nanofibrous scaffolds on DAergic programming fate of two mouse ES cell lines. To this end, we generated, a feeder-dependent and an independent mouse ES cells lines that can overexpress Nurr1 transcription factors (Nurr1-ES). Then, the cells were plated on the PCL/Gelatin nanofibrous scaffolds and differentiate to DAergic-like cells through a standard five-stage protocol. The scanning electron microscopy (SEM) confirmed the attachment and infiltration of Nurr1-ES cells into PCL/Gelatin scaffolds. Quantitative RT-PCR and Immunocytochemistry for midbrain DAergic neurons-specific genes such as tyrosine hydroxylase, dopamine decarboxylase and endogenous Nurr1 showed a higher expression in ES-derived DAergic neurons cultured and differentiate on PCL/Gelatin than cells were cultured and differentiated on gelatin as control. Reverse-phase HPLC result showed that the ES-cells differentiated on PCL/Gelatin scaffolds can efficiently secret dopamine in response to stimulus and the amount of secreted and intracellular dopamine was significantly more in these cells compare to ES-cells cultured on gelatin. In conclusion, our results demonstrated that PCL/Gelatin nanofibrous scaffolds can efficiently support/promote the generation of functional DAergic-like cells from Nurr1-ES cells. The results of this study may have impact in future tissue engineering of midbrain for cell therapy of PD.

Poster Board Number: F-3124

THE TRANSCRIPTIONAL NETWORKS REGULATED BY SOX FAMILY GENES DURING THE TRANSITION FROM EMBRYONIC STEM CELLS TO NEURAL STEM CELLS

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Sox2 is one of the well-known transcription factors involved in cell fate determination as well as in the regulation of embryonic development. With Sox1 and Sox3, this family of genes is important for maintaining the neural progenitor cells in the chick and mouse embryos (Bylund et al. 2003; Graham et al. 2003; Favaro et al. 2009). They maintain neural progenitor cells in an undifferentiated state and repress neuronal differentiation by inhibiting downstream differentiation signals from proneural transcription factors. Later Sox2 was reported as a key transcription factor essential to maintain self-renewal of embryonic stem cells and required for reprogramming induced pluripotent stem cells (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). Recently, Austin Smith and colleagues proposed that ES cells have an innate programme for self-replication that does not require extrinsic instruction (Ying et al., 2008). By blocking the inductive pathways of differentiation with selective small molecule inhibitors SU5402 and PD184352 to inhibit FGF receptor tyrosine kinases and the ERK cascade, respectively, ES cells can be maintained in a 'ground state', meaning a basal proliferative state that is free of epigenetic restriction and has minimal requirements for extrinsic stimuli. To reveal the transcriptional network that underlies the establishment and maintenance of the pluripotent state in mouse ESC and to reveal the detailed changes in the transcriptional network as differentiating the mouse ESC into neural stem (NS) cell, we analyzed and compared the global binding patterns of Sox transcription factors, along with RNA polII, and bivalent histone modifications (H3K4me3 and H3K27me3) under the following distinct conditions: ESC cultured (1) in 2i media (media with 2 inhibitors SU5402 and PD184352); (2) in the presence of LIF and serum/BMP signals; (3) following conversion of mouse ESC to neural progenitors in N2B27 media. We generated mouse ES cell lines with a specific affinity tag knocked into to C-terminus of Sox2. The tag is for the dual purpose of studying both protein-protein and protein-DNA interactions. By applying chromatin immunoprecipitation (ChIP) and Illumina Hi-Seq sequencing, we characterized the binding patterns for Sox family and gained a global view of the signaling pathways and the gene regulatory networks that control/maintain cell fate. Together, our results suggest that (1) many genes in ES cells are pre-bound by Sox2 and epigenetically primed for activation; (2) the binding of Sox1 and Sox3 are strongly associated with active RNA polII binding and bivalent chromatin signatures in NSC, while Sox2 binding is more widely and evenly distributed across the whole genome; and (3) in NSC, although Sox1-3 shared similar targets (e.g. pathways/networks that involved in metabolism, cell cyle regulation via MAPK signaling and Wnt signaling, ubiquitin mediated proteolysis, neuroactive ligand-receptor interaction, axon guidance, and neurotrophin signaling for cellular differentiation and survival etc.), each protein binds a distinct subset of targets. Taken together, these high throughput genomic approaches have enabled us to draft the transcriptional networks regulated by Sox gene family and their sequential action in the maintenance and differentiation of neural progenitors.

Poster Board Number: F-3125

INDUCTION OF HEMATOPOIETIC STEM CELL FROM INDUCED PLURIPOTENT STEM CELL (IPSC) IN SERUM FREE AND FEEDER FREE CONDITION

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Generation of induced pluripotent stem cell (iPSC)-derived hematopoietic stem cell in serum free and feeder free condition in entire processes from reprogramming to hematopoietic differentiation is one of the key factors for the pluripotent stem cell derived-cell transplantation. We reprogrammed 1x104 CD34+ cord blood cells with Sendi Virus (SeV) carrying 4 reprogramming factors (Oct3/4, Sox2, Klf4, c-Myc) and placed on artificial ECM matrix(PronectinF®) coated-6 well plate. Human ES cell-like colonies emerged 21 days after infection in serum free primate ES cell medium supplemented with bFGF. These cells were maintained on PronectinF®-coated dishes for further appraisal of reprogramming. We examined in vitro and *in vivo* differentiation potential of reprogrammed clones via the EB formation and teratoma-formed assay. Then iPSC clones established were seeded on collagen type IV-coated dish in StemlineIImedium contain Insulin-Transferrin-Selenium. To initiate mesoderm differentiation process, iPSC were cultured with 40 ng/mL of BMP4 for 4 days (day 0-3) and then replaced with 50 ng/mL VEGF and 50 ng/mL SCF for day 2 (mesoderm differentiation). Then the cells were cultured with StemlineII medium supplemented with hematopoietic cocktail [25 ng/mL SCF, 10 ng/mL Flit-3, 20 ng/mL TPO, 20 ng/mL IL-6, 20 ng/mL IL-6R] in hypoxic condition 5% O2 on day 6 to follow hemato-endothelial differentiation. After 12 days of culture from initial differentiation, three distinct cell population namely: sac-forming cell clusters, suspended single cells and adherent cells faction merged. After then, we cultured the sac-forming and suspending cell in X-vivo10 base-medium supplemented with hematopoietic cocktail. Interestingly, colony-forming assay showed that the single cells derived from suspended single cell generate CD45+ hematopoietic cells effectively and flow cytometric analysis showed that over 90% of cultured cells expressed hematopoietic cell marker CD45. We report a novel and efficient hematopoitic cell differentiaon methods in entire serum-free and feeder-free culture condition, and these methods will facilitates a safety iPSC-derived hematopoietic cell therapy in future.

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Poster Board Number: F-3126

TARGETING KIDNEY FOR STEM CELL-BASED THERAPY

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Pluripotent human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) can theoretically give rise to all cell types in the human body. With the development of efficient gene targeting technologies in iPSCs, replacement of genetically defective cells with corrected-iPSCs-derived cells becomes possible. Among all target tissues and organs responsible for human disease, kidney and related diseases are among the most pressing health issues in modern societies. The incidence of renal diseases defines an urgent need for development not only of therapeutic approaches but also expanding the general knowledge regarding kidney development and progression of disease. Chronic kidney disease (CKD) is a leading cause of mortality and morbidity in Western countries that is estimated to affect 11% of the adult population. It can progress toward end-stage renal disease (ESRD), which is incurable, often requiring dialysis or preferably renal transplantation. However, the high cost and severe shortage of available transplantable organs greatly limit this possibility for most ESRD patients. It is hoped that the potential of regenerative medicine will contribute to the scientific foundation of novel regenerative approaches in curing ESRD based on cell-based technologies. Yet, there is no established protocol, which can give rise to renal epithelial-like cells fast and efficiently. Thus, differentiation of renal epithelial-like cells and the elucidation of renal progenitor populations represent one of the major bottlenecks in kidney research and its potential translation into stem cell-based therapy approaches. We report here that, using chemically defined media, we were able to drive monolayer human ESCs and iPSCs toward intermediate mesoderm populations, and ultimately renal epithelia-like cells, in a time frame not exceeding 14 days. During the differentiation process, we observed fast induction of Lim1, Pax2, and Osr1, followed by Eya1, WT1, Six2, Ret and Gdnf, and subsequently CDH11 and CDH16. Meanwhile, the epithelial marker EpCAM underwent dynamic down- and up-regulation. We screened additional cell surface markers, and were able to isolate FRZ7+ population after day 8-10 of *in vitro* differentiation, with dramatic enrichment of most above mentioned renal lineage markers. The differentiated epithelial cells showed strong Cytokeratin8 and Cadherin6 staining. We are now injecting the ESCs-derived renal epithelia-like cells into gentamicin-induced acute kidney injury mouse model to validate the functionality. Additionally, considering that kidney progenitor populations have been shown to reside in specific areas of the adult kidney, such as Bowman's capsule, we have performed fractionation of adult human kidney samples. Separation based on CD133 and CD24 surface marker expression allowed for the fractionation of CD133+/24+, CD133+/24-, CD133-/24+, and CD133-/24- populations. Combined with our newly identified cell surface markers, we are currently performing injections of each of the different fractions into the developing mouse kidney E12.5 to evaluate their engraftment capacity. Our current results and ongoing works would provide a simple and efficient methodology for the generation of cell resources with clinical potential for the treatment of ESRD.

Poster Board Number: F-3127

SCL/TAL1 ACTS DURING A TEMPORALLY DEFINED DEVELOPMENTAL WINDOW TO REPRESS CARDIOGENESIS IN HEMATOPOIETIC TISSUES

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Understanding the mechanisms of mesoderm specification into the different lineages during embryogenesis holds a great potential to advance the development of cell-based regenerative therapies for cardiovascular and blood disorders. The divergence of the developmental fates is dictated by transcription factors that induce lineage-specific gene expression programs. The basic helix-loop-helix transcription factor Scl is known as the master regulator for the specification of the hematopoietic fate. We recently discovered that, in addition to its positive effects in promoting the establishment of hemogenic endothelium and hematopoietic stem/progenitor cells development, Scl is also required to repress cardiogenesis in hematopoietic tissues. Strikingly, the absence of Scl led to the development of beating cardiomyocytes in yolk sac vasculature. However, the molecular mechanisms for the cardiac repression have remained poorly understood. Using mouse embryos with tamoxifen inducible deletion of Scl (ERT2-Cre Sclfl/fl), we first showed that Scl is functionally required only during a temporally defined window spanning mesoderm specification and early angioblast development to repress cardiogenesis in hematopoietic tissues. To identify Scl's direct target genes during mesoderm diversification, we determined the genome-wide Scl binding sites in Flk+ mesoderm from embryoid bodies using ChIP-sequencing. This analysis identified > 4600 Scl binding sites throughout the genome, with predominance in inter-genic regions. Comparison with previously published Scl ChIP-seq datasets during later stages of development (HPC7 hematopoietic progenitor cell-line, Wilson et al. 2010, and red blood cells from fetal liver, Kassouf et al. 2010) revealed that the majority of the binding sites are developmental stage specific. Using nearest gene approach to intersect ChIP-seq data with gene expression data showed that 35% of Scl activated and 20% of repressed genes in Flk+ mesoderm were bound by Scl. Similar to later stages of hematopoietic development, robust binding of Scl to key hematopoietic transcription factors downstream of Scl, such as Runx1, Gata1, Gata2, Lyl1, Eto2, Erg, Fli1, Hhex, Gfi1, Gfi1b and Myb was observed during mesoderm specification. Interestingly, genomic regions enrichment analysis of Scl binding sites unique to Flk+ mesoderm showed enrichment for genes implicated in mesoderm formation and heart development, such as Gata4, Gata6, Msx1, Myocd, Nkx2-5 and Tbx5. These results indicate that Scl has a critical function both as an activator and a repressor during mesoderm diversification, and suggest that Scl directly represses cardiogenic transcriptional program during a temporally defined developmental window in order to facilitate the establishment of the hematopoietic system.

Poster Board Number: F-3128

CD49F ENHANCES MULTIPOTENCY AND MAINTAINS STEMNESS THROUGH THE DIRECT REGULATION OF OCT4 AND SOX2

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CD49f (integrin subunit alpha 6) regulates signaling pathways in a variety of cellular activities. However, the role of CD49f in regulating

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the differentiation and pluripotency of stem cells has not been fully investigated. Therefore, in this study, human mesenchymal stem cells (hMSCs) were induced to form spheres under non-adherent culture conditions, and we found that the CD49f-positive population was enriched in MSC spheres compared with MSCs in a monolayer. The expression of CD49f regulated the ability of hMSCs to form spheres and was associated with an activation of the PI3K/AKT signaling pathway. Furthermore, the forced expression of CD49f modulated the proliferation and differentiation potentials of hMSCs through prolonged activation of PI3K/AKT and suppressed the level of p53. We showed that the pluripotency factors OCT4 and SOX2 were recruited to the putative promoter region of CD49f, indicating that OCT4 and SOX2 play positive roles in the expression of CD49f. To determine the role of CD49f in the maintenance of pluripotency, we investigated the role of CD49f in the induction of pluripotency with defined factors. Indeed, CD49f expression was increased during the reprogramming procedures, while the increased level of CD49f expression was significantly decreased upon embryoid body formation in both the induced pluripotent stem cells (hiPSCs) and human embryonic stem cells (hESCs). In hESCs, the knockdown of CD49f down-regulated PI3K/AKT signaling and up-regulated the level of p53, inducing differentiation into three germ layers. Taken together, our data suggest that the cell-surface protein CD49f has novel and dynamic roles in regulating the differentiation potential of hMSCs and maintaining pluripotency.

Reprogramming

Poster Board Number: F-3131

FEEDER-FREE MRNA REPROGRAMMING FOR THE DERIVATION OF CLINICALLY RELEVANT HUMAN IPS CELL LINES

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To date, the widespread adoption of induced pluripotent stem (iPS) cell technology for regenerative medicine and drug screening applications has been limited by the inability to efficiently derive human iPS cell lines that are free from both genomic integration and viral contaminants. Additionally, there has been notable difficulty in the efficient production of human iPS cell lines when using integration-free reprogramming factor delivery with a defined cell culture environment. The advent of mRNA reprogramming methods for the generation of integration-free iPS lines has satisfied the former requirement, but their application in a defined environment has yet to be shown. Here we present data demonstrating efficient derivation of integration-free mRNA iPS cell lines from patient fibroblasts in a feeder-free culture environment. This novel protocol eliminates inherent variability associated with feeder-based reprogramming methods by pairing a defined, xeno-free cell culture medium with pluripotent cell culture attachment substrates. In addition, the protocol incorporates a novel RNA transfection reagent that allows for both a reduction in the number of transfections ultimately required for iPS cell colony establishment, but also a reduction in the amount of mRNA required on a daily transfection basis. Combined, these refinements of mRNA reprogramming provide a defined system to derive iPS cells from patient fibroblast samples that is simple, efficient, as well as integration and feeder-free.

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FIBROBLASTS TO INTEGRATION-FREE IPS CELL LINES: APPLICATION ADVANCEMENTS FOR THE USE OF MRNA TO REPROGRAM HUMAN FIBROBLASTS

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In 2010, Warren, et. al. demonstrated that repeated transfection of fibroblasts with a cocktail of mRNAs resulted in the efficient derivation of integration-free human iPS cells. Our work in 2011 not only reduced the variability of the published protocol, but also increased the reproducibility and efficiency of the system for iPS cell line derivation. Here, we build upon these existing methods and present a broad range of mRNA reprogramming application data that demonstrate: 1) derivation of integration-free human iPS cell lines with minimal numbers of mRNA transfections; 2) inclusion of mature miRNA clusters in a mRNA transfection protocol facilitating iPS cell line derivation from fibroblasts refractory to reprogramming with mRNA cocktail alone; and 3) efficient mRNA iPS cell line derivation across a range of starting target fibroblasts. In addition, we will introduce methods to minimize biological variables that can impact successful iPS cell derivation.

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SISTEMATIC IDENTIFICATION OF MIR-29A TARGETS IN HUMAN BJ FIBROBLAST AND PLURIPOTENT NTERA2 CELLS REVEALS COMPONENTS OF SEVERAL PATHWAYS CONTRIBUTING TO SOMATIC CELL REPROGRAMING

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Despite the great therapeutic potential attributed to induced pluripotent stem cells (iPSs), the molecular basis of somatic cell reprogramming remains largely unknown. Among alternatives for the potentially harmful genetic modifications currently used, microRNAs (miRs) have emerged as a promising field. In fact, transfection of synthetic miRs (pre-miRs) enriched in pluripotent cells, was shown to enhance the efficiency or to directly promote reprograming of somatic cells into iPS cells. Conversely, inhibition of miRs enriched in fibroblasts, by transfection of synthetic anti-miRs, was also shown to enhance reprograming efficiency. Among these, miR-29a transcription was shown to be directly repressed by cMyc during early reprograming and its inhibition (by anti-miR) led to reduced p53 levels (by increasing the protein levels of its regulators p85a and CDC42) and reduced ERK1/2 phosphorylation (by inducing Spry1 expression). Despite these punctual results, a broad and systematic evaluation of how signaling pathways are regulated by miR-29a, is still lacking. With that in mind, we carried a genome-wide identification of miR-29a target. For this, synthetic pre-miR, inhibitory anti-miR and corresponding unspecific control molecules were independently transfected into human BJ fibroblasts and pluripotent NTera2 cells and after 72 hours, gene expression profiles were obtained by oligonucleotide microarrays. Confident targets,

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were identified by comparing to the set of transcripts downregulated by pre-miR transfection and upregulated by the corresponding anti-miR (in both cell lines) to that of predicted targets, showing evolutionary conserved miR binding sites (microrna.org). Transcriptional changes induced by pre- or anti-miR-29a transfection were compared to the changes observed during somatic cell reprograming, to identify signaling pathways potentially relevant for the reprograming process. For this, two distinct fibroblast/iPS transcriptome sets were used (a partially reprogrammed iPS generated by our group and that of Takahashi, obtained by retroviral insertion of OSKM factors). Pathways and biological processes with a statistically significant enriched number of miR-29a targets were identified using DAVID´s Functional Annotation Tool. Among identified pathway (and corresponding targets), we found: anti-apoptotic pathway, upstream of p85a, leading to BAD phosphorylation (IRS1, IGF1R and ERK2); Wnt signaling (CCND2, FZD1, LPPR6, PP3CA, TCF7L1, VANGL1, ROCK1 and ROCK1P1); components involved in chromatin remodeling (including lysine demethylases KDM2A, KDM4A and KDM5A). Finally, central components of active DNA demethylation (TET1 and TDG) were also identified as miR-29a targets and were validated by qRT-PCR. Importantly, Tet1 gene is directly controlled by Oct4 and Sox2 and is induced concomitantly with 5hmC during reprogramming. Moreover, promoters of pluripotency factors are enriched for 5hmC and bound by Tet1. Tet1 knockdown in mouse ES cells decreases total 5hmC levels, increases DNA methylation at the Nanog promoter and reduces its expression, impairing proliferation. We show that several transcripts targeted by miR-29a are components of pathways that contribute to pluripotency, self-renewal and somatic cell reprograming. Our findings contribute to the understanding of how microRNA modulation may contribute for somatic cell reprogramming. Support: FAPESP, CNPq.

Poster Board Number: F-3134

THE ESTABLISHMENT OF NAÏVE HUMAN INDUCED PLURIPOTENT STEM CELLS USING THE SMC4™ MEDIA ADDITIVE

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Pluripotency has been categorized into two undifferentiated states, naïve and prime. Although naïve and prime pluripotent stem cells share many characteristics, it is the naïve cells that most efficiently give rise to chimeras and have high clonality potential. Recent studies have shown that the undifferentiated state of mouse and human pluripotent stem cells can vary based on the culture strategy. As previously described, we have developed a chemical platform comprising a unique combination of four small molecule inhibitors (termed SMC4™) which targets signaling pathways associated with the perturbation of stem cell maintenance and survival. SMC4™ is seen to augment somatic cell reprogramming as well as support the viability, proliferation and self-renewal of established human induced pluripotent stem cells (hiPSCs) in a feeder free and single cell culture system. To investigate the effect of SMC4™ on the undifferentiated state, we compared hiPSC clones generated and long-term cultured in either conventional human embryonic stem cells (hESC) medium or SMC4™ supplemented medium. Global gene expression revealed that although both conventional and SMC4 cultured hiPSCs were more similar to each other as compared to their original fibroblast starting cell line, a significant number of genes displayed greater than 4-fold expression differences between the two conditions. Interestingly, the majority of these genes are known to be involved in cellular differentiation, suggesting that SMC4™ culture supports the undifferentiated state. Moreover, while Xist was modestly reduced in hiPSCs cultured in conventional culture compared to their parental fibroblast cell line, it was significantly repressed in hiPSCs cultured in SMC4™ culture conditions. The expression comparison of all genes located on the X chromosome between hiPSCs cultured under conventional versus SMC4™ medium revealed that the overall expression of X chromosome genes in SMC4™ cultured hiPSCs was significantly higher, suggesting reactivation of the silenced X chromosome. Additionally, when hiPSCs cultured in SMC4™ were returned to feeder cells, they appeared to have a dome-like, compact morphology, similar to mouse ESCs and unlike hiPSCs in conventional medium which display of a flat morphology. Finally, hiPSCs cultured in SMC4™ display a high rate of cloning efficiency and can be single cell sorted at dilute concentrations including 1 cell per well of a 96-well plate. Combined, the properties displayed by SMC4™ cultured hiPSCs and their ability to maintain their undifferentiated state during simultaneous inhibition of the TGFβ and MEK pathways are suggestive of naïve status as described by previous studies. The generation and maintenance of naïve state hiPSCs in SMC4™ provides a platform amenable to pharmaceutical use of pluripotent stem cells: through the enablement of high clonality and improved differentiation potential applications including high-throughput screening and gene targeting are enhanced.

Poster Board Number: F-3135

HDAC6 POST TRANSLATIONAL REGULATION OF MICROTUBULES DURING HUMAN IPS REPROGRAMMING

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Understanding the cytoplasmic mediators of reprogramming during induced pluripotency (iPS) with human cells complements the already growing evidence of the transcription factors and epigenetic chromatin modifications underlying the molecular mechanisms responsible for iPS. Recent evidence supported a role of the cytoplasm and cytoskeleton in mediating the events of nuclear reprogramming, suggesting coordination between nuclear dynamics and cytoskeletal plasticity mediated by the post-translational modifications of microtubules, namely lysine 40 acetylation of α-tubulin. HDAC6, a unique class II histone deacetylase localized in the cytoplasm, has been implicated as a tubulin deacetylase (TDAC) that targets acetylated microtubules, cortactin and heat shock protein (HSP)-90. Evidence using Tubastatin-A (TubA), an inhibitor to HDAC6, demonstrated the loss of centriole acetylation, increased spindle microtubule acetylation, and the prevention of human iPS reprogramming in OSKM transduced HFF1 fibroblasts. Here, we show that HDAC6 shRNA transduced into HFF1 fibroblasts significantly reduces iPS reprogramming in humans. Western blots after shRNA HDAC6 transduction demonstrated dramatic loss of HDAC6 protein detection (>70%) in HFF1 fibroblast cells compared to scrambled shRNA HDAC6. HFF1 fibroblasts transduced with control or HDAC6 shRNA were then reprogrammed for 25 days with the lentiviral STEMCCA iPS plasmid. Results of colony formation after HDAC6 shRNA showed a reduced reprogramming efficiency by ~75% relative to control iPS derivations performed with scrambled shRNA cells. Localization of HDAC6 in control HFF1 fibroblasts using an affinity purified rabbit polyclonal antibody showed cytoplasmic detection with no nuclear staining in interphase cells and centrosome staining at mitosis. After 1μM Tub A for 24 hrs, HFF1 control fibroblast showed similar interphase HDAC6 staining patterns with

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increased acetylated α-tubulin detection in interphase microtubules. However, centrosome HDAC6 detection was much weaker at mitosis and centriole acetylated α-tubulin was eliminated. In HFF1 iPS cells, HDAC6 localization was confined to the cytoplasm with no nuclear staining at interphase but, curiously, no centrosome detection at mitosis. This staining pattern persisted after 1μM Tub A treatment for 24 hrs. Following spontaneous differentiation of HFF1 iPS cells *in vitro* for 30 days, however, nuclear HDAC6 staining was detected in a minority of interphase cells and this expression was perhaps related to the cell cycle stage and/or the cell type formed (i.e. neurites). This pattern was also observed after 1μM Tub A for 24 hrs. Finally, we explored the overexpression of tubulin acetyltransferase enzyme (α-TAT-GFP) in HFF1 fibroblast cells anticipated to increase acetylated α-tubulin detection in transduced cells. We could demonstrate α-TAT-GFP increase in HFF1 fibroblasts with a concomitant increase in acetylated α-tubulin in cytoplasmic microtubules and mitotic spindles, but with an efficiency of less than 5% at all concentrations of the enzyme tested (2-10 μg DNA). Collectively, we conclude that post-translational modification of α-tubulin by HDAC6 represents further evidence that the cytoskeleton, in addition to transcriptional regulation, is essential for iPS derivations and represent an unappreciated example of cytoplasmic post-translational reprogramming. Sponsorship: NIH.

Poster Board Number: F-3136

MECHANICAL STRETCH MODULATES CALCIUM HANDLING DURING DIRECT CARDIAC REPROGRAMMING OF RESIDENT PROGENITOR CELLS IN HUMAN HEART

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Background- Although recent work suggests that defined transcription factors suffice to induce cardiac fibroblasts to undergo cardiomyocyte-like cells in mice, the possible effects of cardiac reprogramming in humans remain to be investigated. Cardiomyocyte calcium handling underlies the mechanical activity of the contractile heart and may be dynamically affected by the mechanical environment. Here, we propose that stretch activation as a physiological stimulus might be essential for cardiac rearrangement to achieve effective contraction and differentiation during direct reprogramming of the targeted cells into cardiomyocytes. Methods- Human cardiac progenitor cells (hCPCs) were isolated and transduced with lentiviral vectors encoding human GATA4, Tbx5, and Mef2C. For cardiomyocytes identification in culture, alpha-MHC promoter directing fluorescent proteins (eGFP) and mitochondoria labeling fluorescent dye, tetramethylrhodamine methyl ester perchlorate (TMRM), were used. Differentiated cardiomyocytes were characterized by immunofluorescent staining, calcium oscillation imaging, and FACS analysis by using signal-regulatory protein alpha (SIRPA), a cell-surface marker specific to cardiac lineage committed cells. Reprogrammed hCPCs by defined factors were kept static or subjected to pulsatile stretch (110% elongation) to test cardiac differentiation. Results- Lentiviral transduction of defined cardiac transcription factors into hCPCs for 7 days was sufficient to obtain a small population of alpha-MHC-eGFP positive cardiomyocytes in culture (<5%). The differentiated cells typically contained mitochondrial membrane potential verified by TMRM uptake as ~15%. Interestingly, we found that ~80% of the undifferentiated hCPCs have already expressed SIRPA, regardless the heterogeneity of isolated hCPCs. By direct cardiac reprogramming, SIRPA expression increased to ~100% of cultured hCPCs. Quantitative RT-PCR revealed that cardiac structural genes and transcription factors include alpha-MHC, beta-MHC, cardiac troponin-T, MLC-2a, and Hand1 expressions were significantly upregulated at 2 weeks after reprogramming; however, these cells never showed spontaneous cardiac beating during the differentiation process. We then tested the possible contribution of cardiac induction by cyclic mechanical stretch along with the factor-based reprogramming. We found that physiologic stretch for 3 days rapidly induced both hCPCs and reprogrammed hCPCs elongation and orientation vertical to stretch direction in culture. This triggers an increased frequency in calcium oscillation for >2.5-fold as well as enhanced cardiac structural gene expressions for >2-fold in reprogrammed hCPCs when compared with the cells without mechanotransduction. However, cyclic stretch significantly suppressed the expression of ryanodine receptor and sarcoplasmic reticulum calcium-ATPase, resulting in reduced calcium transport and gap-junction protein connexin43 expression in reprogrammed hCPCs. Conclusions- Our results indicate that defined cardiac transcription factors are indispensable but not sufficient to induce hCPCs to functional human cardiomyocytes. Mechanical stretch accelerates myocardial lineage commitment of reprogrammed hCPCs but may increase the susceptibility to contractile dysfunction.

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GENERATION OF PRIMED AND NAIVE HUMAN IPS LINES USING CRE-EXCISABLE POLYCISTRONIC VIRAL VECTORS

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We developed and utilized novel polycistronic reprogramming vectors that can efficiently and reproducibly generate karyotypically stable human iPSC from wild type and patient-specific cells generated from accessible tissue sources (both normal and diseased) such as dermal fibroblasts, keratinocytes, cord blood derived endothelial cells and bone marrow derived multipotent stem cells. The lentiviral reprogramming vector consists of a single polycistronic cassette that includes a loxP site allowing for efficient removal of reprogramming cassette following a transient delivery of CRE recombinase; generating virus-free iPSC lines. In order to generate an efficient reprogramming vector we have analyze different combinations of reprogramming factors containing up to seven genetic elements. It is now widely accepted that at least two pluripotent states exist: a "naïve" LIF dependent and a "primed" fibroblast growth factor (FGF) dependent pluripotent stem cell state. Furthermore, naïve stem cells have been shown to integrate into the inner cell mass of the blastocyst and have the capability for germline transmission. An attractive feature of the naïve pluripotent cell state is the ability to generate single cell colonies (cloning); moreover, naive cells have an intrinsic ability for efficient homologous recombination. Taken together, these properties make naïve human embryonic stem cells very attractive for rapid and efficient generation of knock-in and knock-out mutants of human cells. In addition, it is conceivable that human naïve cells may have a better differentiation capacity than primed human ESCs and iPSCs and may be able to efficiently differentiate to specific human lineages that are currently difficult to produce. Using define conditions in combination with our unique excisable polycistronic vectors enable us to generate human naïve ESCs from a variety of human samples. In this work we will discuss the conditions required for efficient generation of CRE-excisable iPSC lines with primed or naïve properties.

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XI-REACTIVATION IN FEMALE HUMAN INDUCED PLURIPOTENT STEM CELLS

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Reactivating a transcriptionally inactive X chromosome (Xi) in somatic donor cells and acquiring two active Xs (Xas) is thought to be one of the epigenetic events in cellular reprogramming toward pluripotency. However, female human induced pluripotent stem cell (hiPSC) lines exhibit considerable variability in X-inactivation status, with Xa/Xi and Xa/Xa lines having been reported. One extensive study reveals that the Xi in the donor cells is not reactivated and thus female hiPSCs retain one Xi. In contrast, a meta-analysis of gene expression profiles in several hiPSC lines indicates that some of the lines have two Xas. Furthermore, in studies generating hiPSCs from X-linked Rett syndrome, some research groups failed to generate Xa/Xa cell lines while others could model the disease with Xa/Xa lines. Reasons for this variability are unknown, but human embryonic stem cell (hESC) lines also exhibit considerable variability in X-inactivation status, and derivation and culture conditions affect epigenetic features of the X chromosome. In this study, we investigated the X-inactivation status of hiPSCs derived using conditions standard in the Yamanaka laboratory. We found that many X-linked genes are highly expressed in our female hiPSC lines compared with other pluripotent stem cell lines, including female hESC lines analyzed. In the female hiPSCs at early passages, XIST, a marker for X-inactivation, was expressed at a high level comparable to that in donor fibroblasts but was greatly down-regulated when assayed at > passage (p)15. Concomitantly with this down-regulation, many X-linked genes were up-regulated. Using several established methods to examine X-inactivation status (e.g., fluorescent *in situ* hybridization with X-linked genes), we found that two Xs are active in our female hiPSCs, consistent with high expression of X-linked genes. Upon differentiation, Xa/Xa hiPSCs silenced one X. In total, 14 out of 16 hiPSC lines (10 four-factor (OCT3/4, SOX2, KLF4 and cMYC) and six three-factor (OCT3/4, SOX2 and KLF4) reprogrammed lines) generated from adult fibroblasts exhibited a significant up-regulation of X-linked genes during cell culture. In addition, the expression levels of X-linked genes in nine of 10 four-factor and three of six three-factor lines at > p15 reached similar levels to those in reported Xa/Xa cells. Thus, we conclude that our reprogramming method frequently produces Xa/Xa hiPSC lines. Reliably generating hiPSCs with the desired Xa/Xi or Xa/Xa pattern is useful in disease modeling, elucidating human X chromosome regulation and clinical applications. The mechanisms of frequent Xi-reactivation in our female hiPSCs are under investigation.

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EPIGENETIC GENERATION OF HUMAN INDUCED PLURIPOTENT STEM CELLS BY RNA REPLICON

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Multiple approaches have now been described for the generation of transgene-free human iPSCs by DNA episomal vectors, floxed lentivirus, adenovirus, Sendai virus, modified mRNA transfection and microRNAs. Of these, RNA based approaches have significant benefits for the epigenetic generation of human iPSCs, including hit-and-run temporal expression and absence of vector integration. However, to date, RNA approaches, including transfection of multiple modified mRNAs, have proven inefficient and rather elusive. Our studies on mRNA transfection determined that the inconsistency of this approach arises primarily due to a short duration of reprogramming factor expression in individual cell level. To address this problem, we generated a self-replicative mRNA system (RNA replicon) for the continuous expression of reprogramming factors. Delivery of RNA replicon into cells results in the continuous, high level expression of reprogramming factors with the efficient and highly reproducible generation of human iPSCs. Importantly, the RNA replicon had been lost in all iPSC clones (>36) examined from multiple starting cells and using different combinations of expressed reprogramming factors. Taken together, these observations demonstrate that RNA replicon is an efficient and highly reproducible epigenetic approach to generate human iPSCs.

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DERIVATION OF HUMAN CLONED EMBRYOS WITH DIFFERENT CHROMOSOME PLOIDY BY NUCLEAR TRANSFER OF HUMAN SOMATIC NUCLEI INTO HUMAN NON-ENUCLEATED OOCYTES

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Therapeutic cloning has tremendous potential in regeneration medicine, however the studies was still limited in derivation of human blastocysts. Last year, Noggle et al. reported the first human triploid ES cells derivation from human SCNT blastocyst by transferring the somatic nuclei into human non-enucleated oocytes, which give a hope to therapeutic cloning. Here we investigated the chromosome ploidy variation of human cloned embryos by nuclear transfer of human somatic nuclei into human non-enucleated oocytes using FISH method. In the micromanipulation, the somatic nuclei were injected into the opposite direction to the oocytes nuclear (normally near the first polar body). The pronuclear position after activation and Y chromosome identification at 8-cell stage were used to analyze the chromosome ploidy. There are two types of chromosome ploidy in human SCNT embryos, including diploid and triploid. 14.3% embryos were not found pronuclear formation. 28.6% embryos were diploid and 57.1% embryos were triploid. In about 60% diploid embryos, the somatic nuclei were not activated successfully and only the pronuclear near the first polar body was found. In these embryos, Y chromosome could not be tested in blastomere biopsied from 8-cell embryos. In other diploid embryos, the somatic nuclei were activated successfully, but the nuclei of oocytes was failed to be activated. In these embryos, the pronuclear

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was far away from the first polar body, and Y chromosome could be tested in blastomere biopsied from 8-cell embryos. The cloned embryos with different chromosome ploidy were derived in this study, however we found none of the cloned embryos with diploid chromosome ploidy developed via 8-cell stage, whereas about 35% the cloned embryos with triploid chromosome ploidy could develop to blastocyst stage. The present results suggested that human non-enucleated oocytes could reprogram the somatic nuclei successfully and form the cloned embryo with diploid or triploid chromosome ploidy, but the diploid embryos seemed not develop to blastocysts. The further study would focus on the reprogramming mechanism by human non-enucleated oocytes.

Poster Board Number: F-3141

CHANGES OF CELLULAR PROPERTIES IN MURINE SKIN-DERIVED MULTIPOTENT PRECURSOR CELLS BY THE TREATMENT OF THIAZOVIVIN AND VALPROIC ACID

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Skin precursor cells (SKPs), originated from dermis, have the ability to differentiate into various types of cells including mesenchymal and neural lineage cells. This cell population also contains property of neural crest stem cells during embryonic development. As the multipotent SKPs show higher expression rates of Klf4, c-Myc and Sox9 than adult fibroblasts do, the cells can be an alternative cell source for the generation of induced pluripotent stem (iPS) cells. Here we presented data demonstrating the effects of two small molecules (valproic acid: HDAC inhibitor versus thiazovivin: ROCK inhibitor) with/without leukemia inhibitory factor (LIF) on change of cellular properties in murine SKPs. The cells from murine back skin dermis were propagated after dissociation into single cells by trypsin, dispase and collagenase treatment in DMEM-F12 (3:1) containing B27 supplement (2%), EGF and FGF (20 ng/ml). SKPs were passaged in the medium supplemented with accutase. At passage 3, either valproic acid or thiazovivin was treated for 72 h with/without LIF. Then, the cells were induced neurogenic, adipogenic, myogenic lineage by standardized protocols. Gene expression level of each treatment group was analyzed by quantitative PCR. The cells have ability of neurogenic, adipogenic and myogenic cell differentiation which are properties of SKPs. The expression level of Snai2, Sox9, Sox10, Ngfr, Oct4, Nanog, Sox2 and Klf4 genes was changed by the treatment of thiazovivin or valproic acid. The expression level of pluripotency-related genes (Oct4 and Nanog) was significantly increased by 10 μM thiazovivin treatment. In particular, combined treatment of LIF and thiazovivin accelerated Nanog expression. On the other hand, valproic acid tended to increase the expression level of neural crest-related genes (Snai2 and Ngfr). Interactions among genes of Wwp2 and Lsd1, and Oct4, Nanog, Sox2, c-Myc, and Klf4 after thiazovivin and valproic acid treatment are under investigation. The results will be presented at the meeting. Our finding shows that the ROCK inhibitor, thiazovivin selectively propagates the SKPs having more potential of pluripotency, these cells may be a suitable cell source for generation of iPS cells. In addition, the HDAC inhibitor, valproic acid may support the differentiation of SKPs into neural crest lineage cells. This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST; Grant number 2011-0027807) and Technology Development Program for Agriculture and Forestry, Ministry for Agriculture, Forestry and Fisheries (MAFF; Grant numbers 109020-3 and 111160-4), Korea.

Poster Board Number: F-3142

REGULATION OF NUCLEAR RECEPTORS DURING CELLULAR REPROGAMMING AND DIFFERENTIATION

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Nuclear receptors (NRs) are ligand-regulated transcription factors that control a number of metabolic, developmental, cellular and physiological pathways. The NR superfamily consists of 48 members in human and 49 in mouse. Because of their abilities to influence many target genes in these pathways by specific ligands, it would be of interest to study regulation of NRs during the process of stem cell reprogramming and differentiation. Using adipose-derived cells as primary starting sources, we profiled expression changes of human (h) and mouse (m) NRs during reprogramming toward induced pluripotent stem (iPS) cells. We found that 38 (h) and 32 (m) NRs are expressed in pluripotent stem cells including embryonic stem and iPS cells. Among these, the majority, 15 (h) and 21 (m), of NRs are downregulated after reprogramming. Nevertheless a significant number, 10 (h) and 5 (m), of NRs are selectively expressed in pluripotent stem cells. The pluripotency-specific NRs include ERRβ, LRH-1, RXRγ, and GCNF. In addition, we studied expression changes of NRs after differentiation into embryoid bodies. The result indicates that the majority of 'downregulated' NRs are upregulated again after differentiation while the majority of 'pluripotency-specific' NRs are downregulated upon differentiation, implying the existence of specific regulatory mechanisms for NR expression. Collectively, our results set the first stage for further investigation of how NRs and their ligands can regulate iPS reprogramming, self-renewal and differentiation of pluripotent stem cells.

Poster Board Number: F-3143

DIFFERENTIAL ROLE FOR OCT4 NUCLEOCYTOPLASMIC DYNAMICS IN SOMATIC CELL REPROGRAMMING AND SELF-RENEWAL OF MOUSE EMBRYONIC STEM CELLS

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Oct4 is a member of the POU family of transcription factors and plays a critical role in both maintenance of the undifferentiated state of embryonic stem (ES) cells and in the reprogramming of somatic cells to induced pluripotent stem (iPS) cells. Oct4 is imported into the nucleus where it functions as a transcription factor; however, the spatiotemporal dynamic behavior of Oct4 remains largely unknown. In the present study, we show that Oct4 is a nucleocytoplasmic shuttling protein. Furthermore, while Oct4 mutants with altered nuclear import/export activity were able to maintain the self-renewal of ES cells, they displayed limited potential for cellular reprogramming. These results indicate that the intracellular localization of Oct4, which is dependent on nucleocytoplasmic shuttling, must be more strictly regulated for cellular reprogramming, suggesting that Oct4 plays differential roles in the self-renewal of ES cells and in somatic cell reprogramming.

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Poster Board Number: F-3144

IPSC CELLS DERIVATION PLASMIDS FROM CD34+ CORD BLOOD CELLS BY NON-INTEGRATING EPISOMAL VECTORS REVEALS GENDER BIAS IN REPROGRAMMING.

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The efficiency of reprogramming of somatic cells into iPSC is known to be affected by the age of the donor, the cell type used, the passage number and the reprogramming method employed, making it difficult to identify parameters that enhance or inhibit cell reprogramming. In order to eliminate the variables of donor age, passage number and viral titer we used a non-integrating episomal vector based method to reprogram human CD34+ cord blood samples and compared the reprogramming efficiency of individual female and male donors as well as pooled male and female CD34+ cord blood samples. Following a single transfection of the episomal reprogramming plasmids we routinely obtain between 25 and 95 TRA-160 positive iPS cell colonies from one million CD34+ cord blood cells after only ten days. Remarkably, our data show that under identical experimental conditions female CD34+ cord blood cells reprogram more efficiently than male cells and that pooled male and female cord blood samples (1:1) also yield more female than male iPS colonies. In order to verify that these iPS cell colonies are bona fide iPS cells we picked and expanded >20 iPSC lines and demonstrate that these express Tra160, Tra1-81, SSEA4, Nanog, Oct4 and sox2, exhibit a normal karyotype and verified that randomly picked iPS cells can in each case form tri-germ lineage teratomas and are integration free. Extensive DNA profiling of 19 iPS cell lines generated from pooled male and female samples further demonstrates that the iPS lines are clonal. We conclude a) that episomal reprogramming of human CD34 cord blood cells occurs rapidly, is highly efficient and yields good quality iPS cells b) that reprogramming is a clonal event and thus amenable to simultaneous multiplexed generation of iPS cells from multiple donors, thereby reducing time and costs and c) that reprogramming occurs more readily in female CD34+ cord blood cells. The reason for this gender bias of reprogramming is at present not clear but may be related to imprinting. *Keywords*: reprogramming; hiPS cells; Cord blood CD34+ cells; karyotype; DNA profiling

Poster Board Number: F-3145

TRAVERSING BETWEEN A PLURIPOTENT AND AN EXTRAEMBRYONIC-LIKE STATE USING RAT STEM CELLS

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Embryonic development is governed by several complex regulatory mechanisms including transcriptional and epigenetic modifications that collectively determine cell fate. During early stages, the cells in the inner cell mass (ICM) are segregated into the epiblast and the primitive endoderm (PrE). The epiblast cells contribute to the embryo proper while the extraembryonic PrE cells contribute to the yolk sac. It was shown in recent studies that cells in the ICM are predisposed towards either the epiblast or PrE state based on the inverse expression of the respective markers Nanog or Gata6 while maintaining similar Oct4 levels, indicating the presence of state-specific precursor populations. The recent isolation of rat embryonic stem cells as well as rat extraembryonic precursors such as XEN-P cells have set stage for elaborate studies regarding these cell fate decisions in vitro. In this study, we present a unique model system which elucidates the intricacy of regulatory networks that guide the transition and lineage commitment between pluripotent and extraembryonic precursor states. We demonstrate that rat embryonic stem cells are transformed to multipotent extra embryonic precursor cells (XMP), when cultured on fibronectin, in media containing PDGF and LIF. These cells retain expression of Oct4 but not Sox2 or Nanog and express primitive endoderm genes such as Gata4, Gata6, Sox17, FGFR2 and PDGFRa. These cells exhibit plasticity to transition back to the pluripotent ES state when transferred into rat ES culture conditions, up to a week. However, after an extended cultivation in the XMP conditions, the cells lose their plasticity and are committed to this state, thereby requiring transfection with exogenous factors to persuade them to the pluripotency. In our lab, we have also derived stem cells from the adult rat bone marrow in the same culture conditions as the XMPs. These cells have a similar PrE precursor-like phenotype and gene expression as XMPs. We have successfully performed the reversal of state between these cells to induced pluripotent stem cells using the traditional four factor approach. These rat IPS cells are similar to rat ES cells by transcriptome comparison using microarrays and protein expression by immunohistochemistry. They can differentiate in vitro to the three germ layer lineages and contribute to teratomas *in vivo*, similar to the rat ES cells. Transcriptome analysis of different stages of these transitions from the pluripotent state to the extraembryonic state and back; throw light on the key regulatory networks acting in a concerted manner to regulate cell fate. This data allows for re-examination of the predefined roles of proteins such as Oct4 or the signaling pathways involving PDGF and FGF4 during lineage segregation and commitment. This system provides valuable insight as it allows continuous monitoring of the back and forth transition of cells among two different steady states namely, the ES and the PrE precursor state. The unique gene expression profile of the primitive endoderm precursor cells and their developmental proximity to pluripotent stem cells make these cells ideal candidates for probing the dynamics of the underlying gene networks.

Poster Board Number: F-3146

UNCOVERING THE EFFECTS OF OCT4 DURING THE EARLY STEPS OF REPROGRAMMING

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Differentiated cells can be reprogrammed into induced pluripotent stem cells (iPSCs) by overexpression of the transcription factors Oct4, Sox2, Klf4, and c-Myc. Among these, Oct4 is the only one that cannot be replaced or eliminated from the reprogramming cocktail. In order to shed light upon the poorly understood reprogramming mechanism, we aimed at exploring the role of Oct4 in the early events of this process. To this end, different somatic cell lines were established from Oct4-inducible transgenic mice. Samples were collected at different time points after Oct4 induction and global gene expression was analyzed by microarray. The differentially expressed transcripts could be divided into two different clusters: the first group comprised genes that were commonly up- or downregulated in all the different cell types, while expression of the second group of genes was only changed in one specific cell type at a time. In summary, our data suggests that during the onset

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of the reprogramming process, Oct4 interferes with each somatic program in a cell type-specific manner while initiating a common pluripotent transcriptional network.

Poster Board Number: F-3147

SPLICING VARIANTS OF TRANSCRIPTION FACTOR NANOG IN THE COMMON MARMOSET MONKEY CALLITHRIX JACCHUS

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Objective: Regenerative medicine focuses on understanding processes involved in naturally occurring cell and tissue regeneration in animals and human in order to exploit this knowledge for new therapies. Here, the focus lays on exploiting the natural function of stem cells and their pluripotency potential to establish tissue regeneration and cell replacement therapy. Due to immune rejection and ethical concerns for using human embryonic stem cells (ESCs), induced pluripotent stem (iPS) cells bear much hope to overcome these obstacles. Unfortunately, the precise mechanism of reprogramming of somatic cells into iPS cells is still not fully understood but can be facilitated by introduction of transcription factors such as SOX2, OCT4, MYC and KLF4. The transcription factor NANOG is not mandatory for reprogramming, however, it is necessary for maintaining ESC self-renewal and is recognized as a quality marker for fully reprogrammed iPS cells in general in mouse and human, but also in the common marmoset monkey Callithrix jacchus. Since there are splicing variants of NANOG known in the human (NANOG 1 and 2), the aim of this study is the identification of putative NANOG variants in the Callithrix jacchus and similarities towards the human NANOG. Furthermore, by analysis of different tissues deduction of possible function of NANOG splicing variants can be attempted. The appearance of splicing variants could possibly enhance our understanding in reprogramming and improve reprogramming rates and quality of iPS. Material and Methods: For the analysis, based on database in-silico analysis, ESCs, iPS cells and various tissues of the common marmoset were screened by RT-PCR to identify NANOG splicing variants. Open reading frame and splicing variants analogue to the human could be amplified with different oligonucleotide pairs. cDNA fragments of correct size were subcloned into the pCDNA 2.1 vector and sequenced (Sanger). Results: Surprisingly, in-silico NANOG genebank sequence differed from our sequenced version by 9 % representing 49 amino acids. Two NANOG splicing variants with a size difference similar to the human could be detected. Screening of tissues, ESCs and iPS cells displayed the presence of only the large variant in adult ovary, which is in contrast to the human ovary where NANOG expression can only be detected in the fetal ovary and is abolished as oogonia enter meiosis. Only the small fragment was found in the ESCs and fragments both sizes in the iPS cells. No NANOG expression could be found in the testis, mesenchymal cells and skin fibroblasts.

Poster Board Number: F-3148

UNDERSTANDING THE TRUE NATURE AND POTENTIAL OF PARTIAL IPS STATES

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Somatic cells can be successfully reprogrammed to an induced Pluripotent Stem (iPS) cell state that closely resembles embryonic stem (ES) cells. During somatic cell reprogramming a large number of cells fail to fully reprogram to an iPS state and such cell types are often referred to as partial iPS cells. Partial iPS cells have received relatively little attention due to their differential gene expression in relation to ES cells, as a consequence the full potential of partial iPS cell types necessitates further characterization. Previous reports document that partial iPS cells fail to activate endogenous Sox2, Oct4 and Nanog expression and that their partially reprogrammed state remains dependent on the expression of the reprogramming factors. Nevertheless, these partially reprogrammed iPS cell lines could be maintained for prolonged periods of time without further progression to a fully reprogrammed state. We have reprogrammed embryonic fibroblast cells through the over expression of the Yamanaka factors (Sox2, Oct4, c-Myc and Klf4) and characterized all colonies and subsequent clonal cell lines irrelevant of cell morphology. This has enabled the analysis of a multitude of partially reprogrammed cell types, which fail to become factor independent mature iPS. We have observed that every partial iPS clone reactivated endogenous Nanog and Oct4 expression, however, each line remained dependent on the expression of the reprogramming transgenes. All clones were examined on the basis of global gene expression and this information utilised to map the diversity of partial iPS states in relation to mature iPS and ES cells. Notably, we have found that the global gene expression and subsequent phenotype of partial iPS cells does not correlate with their expression levels of the reprogramming factors. Every partial iPS cell line has the potential to efficiently generate differentiated cell types representative of all three germ layers *in vivo*, and can be differentiated to terminally differentiated cell types in vitro. Although the partial iPS clones exhibit demethylation of the Nanog and Oct4 promoters we have successfully identified a core set of ES associated genes that remain transcriptionally silent. This core set comprises of genes that are most resilient to reactivation during somatic cell reprogramming, representing an epigenetic block to generating fully reprogrammed iPS cells. We have subsequently shown that this epigenetic block can be rapidly overcome through manipulation of global epigenetic regulators. In summary, this study comprehensively characterizes a multitude of partial iPS states and demonstrates that these cells are competent to differentiate to all three germ layers. Furthermore, we have identified a coherent cluster of transcriptionally silent genes responsible for the partial iPS state and devised an efficient method to efficiently activate expression of these genes to push partial iPS cells to a mature iPS state.

Poster Board Number: F-3149

A CAENORHABDITIS ELEGANS MODEL FOR *IN VIVO* DIRECT CELL REPROGRAMMING

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The steps involved in reprogramming a differentiated cell directly into another cell type (direct cell reprogramming) are not well understood in vitro and have rarely been attempted or studied in vivo. We are developing a model to study direct cell reprogramming in vivo using the nematode *C. elegans* by taking advantage of the known transcriptional cascade that activates embryonic endoderm specificiation and differentiation. In contrast to other endoderm-directing factors, such at the END-1 GATA factor, we found that ectopic expression of a single endoderm-specific GATA transcription factor, ELT-7, can promote ectopic expression of an endoderm reporter gene in fully differentiated cells in larvae. This ectopic expression is quickly lost in most cells, which appear either to maintain their normal differentiation state or die. Muscle cells located in the pharynx, however, maintain the marker expression, contain birefringent granules that are normally specific markers of

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the differentiated intestine, and express the late intestinal brush border protein IFB-2, a marker of late gut differentiation. We are examining pharyngeal muscle-specific markers to assess whether these cells undergo bona fide transdifferentiation. The known cell lineage and cellular anatomy of *C. elegans* will allow us to monitor this process at single cell resolution. These experiments may reveal basic principles involved in direct cell reprogramming and may guide studies in other systems.

Poster Board Number: F-3150

REPROGRAMMING ADULT PROXIMAL TUBULE CELLS TO EMBRYONIC NEPHRON PROGENITORS

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Mammalian kidney organogenesis commences with the specification of the metanephric mesenchyme (MM) and outgrowth of the ureteric bud (UB) from the nephric duct. A series of reciprocal interactions between these two mesodermal populations drives the continued outgrowth of the UB which begins branching upon contact with the MM. Around each branch tip, the MM condenses to form cap mesenchyme (CM) which exists as the source of nephron progenitor (NP) cells. This self-renewing population of kidney stem cells give rise to all nephron segments, except for the collecting ducts, and can be identified by Six2 and Cited1 expression. Following induction, NP cells exhibit substantial genetic and morphological changes, undergoing a mesenchymal-to-epithelial transition to produce the precursory structure to a single nephron, the renal vesicle (RV). Subsequent cell recuitment and elongation of the RV forms the mature nephron. Before or shortly after birth, a final wave of nephrogenesis exhausts the NP population, making it impossible to generate new nephrons after this period. This lack of regenerative capacity underpins the gradual decline in renal function with age and disease, including chronic kidney disease. What we now appreciate is that individuals with low nephron endowment, caused by factors such as malnutrition and premature birth, are susceptible to renal disease in later life due to this inability to generate new nephrons. In the current study, we investigated whether adult proximal tubule cells could be reprogrammed to a NP state via the forced re-expression of genes that define the progenitor population. Using the human proximal tubule cell line (HK2) as a model system, various combinations of 15 genes individually cloned into lentiviral vectors were screened for their ability to induce i) an epithelial-to-mesenchymal morphological change, ii) expression of the NP-specific protein, Cited1, iii) re-expression of key NP genes and iv) integration into the progenitor compartment in an *ex vivo* functional assay (the 'recombination assay'). Following these validation stages, we identified a combination of genes (Osr1, Six1, Six2, Hoxa11, Eya1 and Snai2, or 'Pool-8') that, when transduced into HK2 cells cultured in valproic acid-containing media, were sufficient to induce a transcriptional profile resembling that of NP cells. Furthermore, approximately 0.875% of HK2 cells subjected to the reprogramming process incorporated into progenitor compartments of *ex vivo* embryonic kidney cultures. In contrast, control HK2 cells cultured under identical conditions had an incorporation rate of just 0.05%. These results represent the first evidence that it may be feasible to regenerate the NP population and have seen us extend this study into primary cell models. Preliminary experiments suggest that reprogramming may also be achieved in primary human proximal tubules cells, which showed a large increase in NP-specific genes, Six2 and Cited1 following transduction with Pool-8 lentiviruses. By furthering our understanding of this process, we hope to not only realise the possibility of re-initiating nephron formation post-birth, but to also develop a means by which nephron endowment can be prolonged.

Poster Board Number: F-3151

HISTONE METHYLTRANSFERASE DOT1L IS A BARRIER TO SOMATIC CELL REPROGRAMMING

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Generation of induced pluripotent stem cells (iPSCs) from differentiated cell types by somatic cell reprogramming involves global epigenetic remodeling. To address how chromatin-modifying proteins influence the reprogramming process, we used shRNAs to target genes in DNA and histone methylation pathways, and have identified positive and negative regulators of iPSC generation. Specifically, inhibition of Dot1L, the H3K79 histone methyltransferase, either by RNAi or a small molecule inhibitor accelerated reprogramming, significantly increased the yield of iPSC colonies, and substituted for Klf4 and c-Myc in the reprogramming cocktail. Inhibition of Dot1l early in the reprogramming process is associated with a marked increase in two alternative reprogramming factors, Nanog and Lin28. In loss-of-function experiments, we show that Nanog and Lin28 play essential functional roles in the enhancement of reprogramming by Dot1L-inhibition. Genome-wide analysis of H3K79me2 distribution by ChIP-sequencing revealed that fibroblast-specific genes associated with the epithelial to mesenchymal transition lose H3K79me2 in the initial phases of reprogramming. Dot1L inhibition facilitates the loss of this mark from genes that are fated to be repressed in the pluripotent state. These findings implicate specific chromatin-modifying enzymes as barriers to reprogramming, and demonstrate how modulation of chromatin-modifying enzymes can be exploited to more efficiently generate iPSCs with fewer exogenous transcription factors.

Poster Board Number: F-3152

INDUCTION OF OSTEOCHONDROPROGENITORS FORMATION DURING TRANSCRIPTION FACTOR MEDIATED REPROGRAMMING PROCESSES

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The potential of stem-cell based skeletal tissue engineering has been limited by its heterogeneous and uncontrolled differentiation. Osteo-chondroprogenitors uniquely co-expressing Sox9 and Runx2 with dual differentiation potential to become chondrocytes and osteoblasts is an ideal candidate for skeletal tissue regeneration. Therefore, developing effective strategies to generate sufficient quantities of osteo-chondroprogenitors are essential. Toward this, we take advantage of two lineage reprogramming approaches - one is induced pluripotent stem (iPS) cells generation using osteoblasts and another one is the induction of chondrocyte lineage from skin fibroblasts. The selection of osteoblasts is based on the fact that it is originally derived from osteo-chondroprogenitor lineage and the stochastic events of iPS induction might revert osteoblasts first to their progenitor state before becoming pluripotent. The second approach is based on a previous report using three transcription factors (Sox9, Klf4 and c-Myc) to reprogramme skin fibroblasts into chondrocyte lineage. Our aim is to examine whether osteo-chondroprogenitors would be formed during the

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two reprogramming processes using Sox9-EGFP knock-in mice as a reporter. Our data showed that osteoblasts can be reprogrammed to iPS cells with pluripotency as shown by their ability to form teratomas and contribute to chimeric embryos. Transient appearance of Sox9/Runx2+ve cells was observed during iPS reprogramming. Similar phenomenon was observed during induction of chondrocyte lineage from skin fibroblasts by Sox9, klf4 and c-Myc, raising the possibility that osteochondroprogenitor could be formed during these two transcription factor-mediated reprogramming processes.

Poster Board Number: F-3153

INSIGHTS INTO THE GENETIC AND EPIGENETIC CHANGES UNDERLYING REPROGRAMMING

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With the advent of reprogramming came a powerful approach for answering fundamental questions in developmental biology as well as the ability to explore new possibilities in regenerative medicine. However, in order to fully capitalize on induced pluripotent stem cell (iPSC) technologies, whether for future clinical use, disease modeling, or as an avenue to study development, we need to better understand the molecular mechanisms underlying how a cell reverts back into a pluripotent state and the molecular consequences that may exist following this type of cellular conversion. Accumulating evidence has shown that human iPSC lines display genetic and epigenetic variations, when compared to one another or human embryonic stem cells, such as methylation aberrancies, variable X-inactivation patterns, and somatic coding mutations. The mechanisms underlying the development of these genetic and epigenetic variations during reprogramming and their functional consequences remain largely unclear. To examine these important questions, we have performed reprogramming studies on samples isolated from monozygotic twins. As previous findings have demonstrated epigenetic divergence during the lifetime of monozygotic twins that results in gene expression changes and phenotypic variance, our study enables the investigation of the effect of reprogramming on exact genomes with divergent epigenomes. From an existing University of California San Diego Twin/Sibling/Pedigree cohort, we have generated iPSCs from fibroblasts derived from 3 pairs of genotyped monozygotic twins (all Caucasian females greater than 50 years of age), using both integrative (retroviral-mediated) and non-integrative (sendai virus) approaches. To distinguish between genetic versus culture-condition or reprogramming method-induced variability that may occur, 3 iPSC lines were generated from each individual using each reprogramming approach (6 individuals x 3 iPSC lines x 2 methods = a total of 36 iPSC lines), and were characterized using standard methods (e.g., pluripotency marker expression, germ layer differentiation, karyotyping). Chromatin mapping, RNA-Seq, eQTL, X-inactivation and imprinting analysis, as well as directed differentiation assays of these samples are ongoing. The analysis of these parameters in iPSCs derived from monozygotic twins enables us to examine multiple questions regarding DNA methylation variability and heritability, including but not limited to how reprogramming affects the methylation changes that may be present in genetically identical starting populations, if certain epigenomic changes are selected for/against during reprogramming as well as after (or can affect) differentiation, and how these epigenomic changes relate to genotypic and phenotypic variance.

Poster Board Number: F-3154

TLX MAINTAINS NEURAL STEM CELLS BY REPRESSING THE NEUROGENIC PATHWAY

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Tailless (Tlx, NR2E1) is an orphan nuclear receptor, which is expressed in the periventricular neurogenic zone during mouse embryonic development and expressed exculsively in astrocyte-like B cells of the subventricular zone (SVZ). Tlx acts as a key regulator of neural stem cells (NSCs) expansion and brain tumor initiation from NSCs. Overexpression of Tlx antagonizes age-dependent exhaution of NSCs in mice and leads to migratrion of stem/progenitor cells from their natural niches. The increase of NSCs persists with age and leads to efficient production of newborn neurons in aged brain tissues. Here, we demonstrate that ectopic expression of Tlx using BAC-based technology and Cre/Loxp system induces impaired generation of mature neurons not only in the developing brain but also in the SVZ, one of the largest germinal zones of sustained neurogenesis in the adult mice. In the developing brain of ectopic-expressed Tlx mutant mice, new born neurons are decreased. Furthermore, NSCs corresponding to astrocyte-like type B cells in the adult SVZ give rise to transient amplifying type C cells, which in turn differentiate into type A cells that migrate to the olfactory bulb and then become mature local interneurons. In the ectopic-expressed Tlx mutant mice , more immature neurons from new born cells are significantly observed in the olfactory bulb while less mature neurons with abbrent morphology changes are found in the neural linage differentiated NSCs *in vitro*. This study elucidates that Tlx maintains the stemness of NSCs by repressing the neurogenic pathway.

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PROGRAMMING FIBROBLASTS TO HEMOGENIC ENDOTHELIUM BY DEFINED FACTORS

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Once committed, the differentiated state of a cell is normally stable and can be inherited through cell division. Under certain conditions cell fate can, however, be modified or reversed. Recent work has shown that mouse and human fibroblasts can be reprogrammed to pluripotency or directly converted to unrelated somatic cell fates by combinations of transcription factors (TFs). This raised the question whether TFs could induce somatic stem cell multipotency and self-renewal. Here the requirements to convert fibroblasts to hematopoietic stem cells (HSCs) were investigated using combinatorial TF approaches. We hypothesized that combinatorial expression of HSC-specific TFs could program fibroblasts to recapitulate HSC generation through hemogenic endothelium. We expressed combinations of TFs in mouse embryonic fibroblasts derived from huCD34tTA/TetO-H2BGFP embryos. This reporter system specifically marks HSCs and endothelial/hematopoietic progenitors. Starting from a pool of eighteen candidate genes, we used an iterative process of elimination to define the minimal pool of three and optimal four TFs to induce huCD34-positive colonies. Transduction

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of the identified set of TFs elicits hematopoietic and endothelial-associated gene expression in fibroblasts. Remarkably, the gene expression profile of induced huCD34-positive cells clustered with bona fide bone marrow HSC populations isolated from adult mice. Transcriptional reprogramming is accompanied by the induction of hemogenic colonies that lose adherence and express hematopoietic (c-Kit and CD45), and endothelial (VeCadherin, CD31, CD34, Tie2, Ulex europaeus lectin biding) markers of hemogenic endothelium and emergent HSCs. In addition, induced hemogenic endothelial cells are able to uptake acetylated-LDL. Interestingly, our results show that hemogenic colonies originate from a progenitor cell-type that, in addition to the activity of the huCD34 reporter, express Prominin-1 and high levels of Sca-1. Hence, we provide evidence that the initiation of a complex developmental process can be programmed *in vitro* by a small combination of TFs. Collectively, our results support the view that programming HSCs is multistep and underscore the requirement of progenitors and endothelial intermediates for HSCs generation. Inducing HSCs from fibroblasts may have important implications for hematopoietic development, disease modeling and generation of multipotent tissue-specific stem cells for cell replacement therapies.

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ENHANCED FOOTPRINT-FREE REPROGRAMMING USING SECOND-GENERATION RNA COCKTAILS

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Transient transfection of cocktails comprising mRNAs and/or miRNAs offers perhaps the safest available approach to cellular reprogramming and fate direction, and could well become the method of choice as these applications reach the clinic. While this technology obviates the screening and cleanup steps entailed by other footprint-free reprogramming systems, rapid turnover of RNA in the cytoplasm implies the need for multiple transfections, increasing the hands-on time required to induce cells with the phenotype of interest. For example, as many as 18 consecutive daily transfections are employed when generating iPSCs using mRNA. In addition, it has so far proved challenging to carry out RNA-mediated reprogramming under feeder-free conditions, or in cell types which transfect poorly, such as blood cells. These drawbacks to the methodology may be alleviated in the future through improved delivery techniques or by modifications to the RNA to increase its specific activity and functional half-life. Another strategy is to exploit novel reprogramming factors and factor combinations to improve the efficiency and kinetics of fate conversion. Focusing on the latter approach, we evaluated several candidate factors which have been reported to promote iPSC induction in the literature, and consequently were able to define novel RNA cocktails which substantially enhance reprogramming performance compared to current protocols. Our results suggest that there remains ample scope for increasing the convenience and power of this promising new approach to cell fate manipulation.

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SPONTANEOUS RESCUE OF TRISOMY 21 FIBROBLASTS DURING REPROGRAMMING TO PLURIPOTENCY

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The advent of reprogramming technology has opened up many new avenues for the study of disease. The ability to take fibroblasts from a diseased patient, reprogram them into induced pluripotent stem cells (iPSCs), and then differentiate those iPSCs into the cell type affected in the patient's disease is leading to many new insights into the etiology of numerous diseases. This approach is based on the assumption that the genetic aberrations that cause the disease in the patient will be present in the iPSCs and can drive disease-relevant cellular phenotypes in the differentiated derivatives. We have made the surprising observation that the reprogramming of non-mosaic Down Syndrome (trisomy 21) fibroblasts leads to spontaneous loss of one copy of chromosome 21 in a subset of the resulting iPSC clones, which become seemingly normal, euploid cells. SNP genotyping of trisomic fibroblasts and disomic iPSCs shows that they are derived from the same person, eliminating the possibility that the disomic clones were derived from contaminating euploid cells from another individual. In addition, the spontaneous loss of chromosome 21 occurs regardless of the method used to reprogram the cells. Current studies are aimed at characterizing the differences between disomic and trisomic clones and investigating the mechanism of the conversion of trisomic fibroblast cells into disomic iPSCs. The generation of both trisomic and disomic iPSC lines from the same individual provides us with an unprecedented opportunity to study disease mechanisms at work in Down Syndrome, with the disomic lines serving as the ideal control for the trisomic lines. In the future, these findings may suggest novel therapeutic strategies for the treatment of Down Syndrome.

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MECHANISM OF LET-7 INHIBITION-INDUCED PLURIPOTENT STEM CELL REPROGRAMMING

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Fibroblasts can be directly reprogrammed into induced pluripotent stem (iPS) cells by expressing the pluripotency genes OCT4, SOX2, KLF4, and c-MYC. Without c-MYC, reprogramming efficiency is greatly reduced. LIN28, a protein that regulates processing of the let-7 family of microRNAs, also promotes reprogramming. However, the key genes that function downstream of these reprogramming factors and the mechanism by which they act to direct conversion of fibroblasts into iPS cells remain poorly understood. We found that inhibition of let-7 promoted reprogramming with OCT4, SOX2, and KLF4 to a level of efficiency comparable to reprogramming with OCT4, SOX2, KLF4, and c-MYC. In addition, downregulation of let-7 was necessary for reprogramming. We have identified a single

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target of let-7 regulation that increases reprogramming efficiency, similar to let-7 inhibition. The function of this target gene promises to reveal novel mechanisms of reprogramming.

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NOVEL ALKALINE PHOSPHATASE LIVE STAIN TO IDENTIFY AND ISOLATE EMERGING IPSCS

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Differential expression of Alkaline Phosphatase (AP) in pluripotent cells has provided a useful tool to identify emerging pluripotent colonies during the process of somatic reprogramming. So far, most available AP substrates are toxic to the cells and once stained; the cells cannot be propagated further. We have previously reported the development of a novel Alkaline Phosphatase Live Stain (AP Live Stain) that specifically stains pluripotent stem cells while preserving cell integrity. Here we demonstrate the use of the AP Live Stain to monitor and select colonies during reprogramming. AP Live Stain was used in multiple reprogramming experiments using the CytoTune™-iPSC Sendai Reprogramming Kit. AP Live Stain was applied on the master reprogramming plates and utilized during the initial colony selection process. Of the several hundred colonies observed on the dishes, some colonies had more robust and uniform AP staining than others. Colonies that were morphologically accurate with robust, homogeneous AP Live Staining were manually picked and transferred to individual wells. All the AP Live Stain selected colonies expanded normally while retaining normal morphology and pluripotent marker expression. Clones that were chosen for further expansion and characterization were karyotypically normal and pluripotent based on marker expression and ability to differentiate into cell types representative of the three germ layers. Based on these results, subsequent sets of reprogramming experiments solely utilized AP Live Staining for identification and selection of emerging iPSC colonies. AP Live Stain was further used in feeder-independent derivation of iPSCs. All AP Live Stain selected clones survived and retained appropriate morphology and marker expression. Selected clones from subsequent experiments in feeder dependant and feeder independent culture conditions were further expanded and shown to possess normal karyotypes, marker expression and tri-lineage differentiation potential. These results demonstrate the utility of AP Live Stain during reprogramming to monitor and select bona fide reprogrammed clones. AP Live Staining provides an easy alternative to antibody methods for early monitoring and screening of cells during reprogramming. The utility of this dye in reprogramming other somatic cells and utilization of other reprogramming methods are currently being investigated.

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PROTEIN STRUCTURE INITIATIVE ON STEM CELL BIOLOGY

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Understanding mechanisms of transcriptional regulation is a major goal in molecular biology. Most molecular events in controlling gene expression and cell programming require transient multi-protein complexes that act in response to different physiological signals to interpret enhancers and promoters found in the DNA. Much progress has been made in structure and function analyses of individual components of these macromolecular machines, but few detailed mechanisms are known for multi-component assemblies. We have worked on this problem by focusing on the critical transcription factors essential to maintain pluripotency of embryonic stem cells and to differentiation to other type of cells. Among these transcription factor complexes, we will report on human Sox9-DNA complex structure at 2.8 Å, showing the structural basis of DNA recognition by Sox9, which is essential in male sex determination and chondrogenesis. The Sox9 DNA binding domain includes many of Campomelic dysplasia-causing mutation sites and the structure at atomic resolution suggests a part of molecular insight into the pathogenesis. Crystallographic studies on human Oct4 and Sox2 with DNA, and human Tbx5 and Mef2c with DNA complexes also will be presented. We acknowledge that the project is a collaboration with the Joint Center for Structural Genomics, directed by Ian Wilson.

Poster Board Number: F-3161

REPROGRAMMING OF MATURE B CELLS INTO ERYTHROID LINEAGE

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Direct reprogramming of somatic cells into another is a powerful tool to generate autologous cells for regenerative medicine. In hematopoietic system, induction of erythrocytes by direct reprogramming is an attractive approach for obtaining patients' own red blood cells for transfusion. Previous studies have shown that hematopoietic lineage-committed progenitors retain plasticity and can be reprogrammed into other lineages by ectopic cytokine signaling or forced expression of lineage-specific transcription factors. Based on these findings, we hypothesized that mature differentiated B cells could be reprogrammed into other lineages by modulating their transcriptional program. To test this hypothesis, we attempted to reprogram terminally differentiated mature B cells into erythroid lineage by transducing various transcription factors using retrovirus, and to define minimal set of factors required for erythroid reprogramming. For reprogramming, we expressed transcription factors related to erythroid differentiation such as GATA-1 and SCL. In addition, we used transcription factors expressed in immature hematopoietic cells such as GATA-2, Hes-1 and Evi-1, hoping that they facilitate the reprogramming process, and CCAAT/ enhancer binding protein α (C/EBPα) known to be required for efficient reprogramming of mature B cells. We first purified mature B cells of C/EBPα-ER transgenic (Tg) mice, in which activation of C/EBPα can be controlled by 4-hydroxytamoxyfen (4-HT), and stimulated them with LPS and anti-CD40 antibody for 24 hours. Activated B cells were transduced with the above listed genes using retrovirus, and plated in methylcellulose for colony assays. Interestingly, erythroid colonies highly similar to burst forming unit-erythroid (BFU-E) showed up after 7-days of culture. Of three colonies examined, GATA-1-ER was commonly integrated in all three clones, whereas SCL was integrated in two clones. These clones retained rearrangement of immunoglobulin heavy chain gene, indicating that these colonies originated from differentiated B cells. These data suggested that GATA-1 and SCL were essential for erythroid reprogramming of B cells, and we next tested the requirement of each single transcription factor in the reprogramming process by transducing GATA1 or SCL separately or in combination into B cells from C57BL6/J or C/EBPα-ER transgenic mice. Interestingly, erythroid colonies were formed by GATA-1 alone, but co-introduction of SCL and C/EBPα significantly enhanced the efficiency of erythroid conversion. Lastly, we analyzed the expression of lineage-specific genes in the converted colonies by RT-PCR. The analysis revealed that the repro-

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grammed erythroid colonies expressed erythroid-specific genes such as Epo receptor, Fog1 and β-globin, while myeloid-specific genes such as G-CSF receeptor, M-CSF receptor and GM-CSF receptor were not expressed. In conclusion, this study indicates that GATA1 in combination with SCL and C/EBPα efficiently converts mature B cells into erythroid lineage. This establishes a technical foundation for erythroid reprogramming, which will be a valuable strategy for production of mature red cells for transfusion.

Poster Board Number: F-3162

THE OPPOSING FUNCTIONS OF MYOCYTE ENHANCER FACTOR 2 PROTEINS AND CLASS IIA HISTONE DEACETYLASES PROVIDE A RHEOSTATIC CONTROL FOR SOMATIC CELL REPROGRAMMING

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The antagonism between myocyte enhancer factor 2 (MEF2) and class IIa histone deacetylases (HDACs) functions as a module that orchestrates tissue specification in embryogenesis. Here we describe that this module also controls somatic cell reprogramming by defined factors. MEF2 proteins and class IIa HDACs have low expression in somatic cells but rise asynchronously during reprogramming. MEF2 proteins tend to block the process by antagonizing Klf4, consequently preventing the phase of mesenchymal-to-epithelial transition. Class IIa HDACs have the opposite function by counterbalancing the activity of MEF2, thus creating a modulating rheostat. In addition, calcium/calmodulin dependent protein kinases (CaMKs) fine-tune this rheostat by promoting the nuclear export of class IIa HDACs, which impairs reprogramming in the initial phase but later facilitates it. Our results reveal an unexpected role of the MEF2 proteins/class IIa HDACs/CaMKs axis in reprogramming, and suggest that certain developmental signals must be switched on and off to acquire pluripotency.

Poster Board Number: F-3163

GATA4 BLOCKS SOMATIC CELL REPROGRAMMING BY REPRESSING NANOG TRANSCRIPTION

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Somatic cells can be reprogrammed to induced pluripotent stem cells (iPSC) by the transgenic expression of three transcription factors with or without Myc: Oct3/4, Sox2 and Klf4 (OKS/OKSM). iPSC can then be differentiated towards different cell lineages. However, somatic cells can also be directly reprogrammed to other cellular types by the expression of lineage-specific transcription factors. During the establishment of a protocol to differentiate mouse embryonic fibroblasts (MEF) to definitive endoderm by co-expression of OKS with endoderm-specific transcription factors, we observed that Gata4 was able to completely block Nanog expression during iPCS formation. This effect was Gata4-specific and was not the consequence of committing to an endoderm lineage (extraembryonic, primitive, parietal, visceral nor definitive). Absence of Nanog was correlated with absence of pluripotency markers, while expression of other markers linked to iPSC formation, such as those involved in mesenchymal-epithelial transition (MET), were as expected. Addition of a retrovirus expressing Nanog reversed the observed fenotype. Using comparative genomics we identified two major evolutionary conserved regions (ECR) located 9 kilobases upstream of the start site of Nanog transcription. Both ECR contained bona fide binding sites for Gata factors. We show that Gata4 binds to both sequences and repress transcription of Nanog in mouse embryonic stem cells (mESC). We concluded that Gata4 might directly repress Nanog expression during iPSC formation and in mESC.This results highlights the inter-regulatory networks controlling induction and maintenance of cell lineage choices involved in segregation of the primitive endoderm and epiblast from the inner cell mass.

Poster Board Number: F-3164

A SMALL MOLECULE SCREEN REVEALS NOVEL REGULATORS OF DIRECT CARDIAC REPROGRAMMING

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Reprogramming of adult cells into pluripotent cells or directly into alternative adult cell types holds great promise for regenerative medicine. We recently reported that cardiac fibroblasts, which represent 50% of the cells in the mammalian heart, can be globally reprogrammed directly to adult cardiomyocyte-like cells with the addition of Gata4, Mef2c and Tbx5 (GMT). Here, we report the use of chemical biology to probe the mechanism of cardiac reprogramming in an effort to improve the technology. We performed a small molecule screen to identify chemicals that enhanced or repressed the conversion of fibroblasts to induced cardiomyocytes (iCMs). Several chemicals were identified that repressed GMT-mediated reprogramming, and these have led to discovery of growth factors that can positively regulate the efficiency of cardiomyocyte generation from fibroblasts. In addition to increasing efficiency, this discovery has provided insight into the mechanisms involved during the transdifferentiation into iCMs. Details of the screen will be presented, along with evidence for enhancement of the reprogramming technology.

Tissue Engineering

Poster Board Number: F-3165

ATTACHMENT AND THE PROLIFERATION OF HUMAN AORTA ENDOTHELIAL CELLS ON SIS/PLGA FILM

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The endothelium is a multifunctional tissue that actively participates in the maintenance of cardiovascular homeostasis. Small intestinal submucosa (SIS) is an acellular biomaterial usually derived from the jejunum of pigs. We fabricated hybrid polymeric films containing PLGA and various concentration of SIS to confirm whether the SIS affect the human aortic endothelial cells (HAECs) behavior and find suitable concentration of SIS in the hybrid film for HAECs culture. The preservation of cell phenotype on the SIS/PLGA films was confirmed by the presence of cell-specific markers, including eNOS, PECAM-1, KLF2 and VE-cadherin for HAECs at both protein and transcription levels using immunocytochemistry and RT-PCR, respectively. The results indicated a favorable interaction between vascular cells and SIS/PLGA films. These data confirmed the potential of this novel hybrid films in vascular tissue engineering. This research was supported by WCU (R31-20029) and SCRC (SC4110).

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EFFECTS OF SILK CONTENT ON THE ATTACHMENT AND THE PROLIFERATION OF HUMAN AORTA ENDOTHELIAL CELLS ON SILK/PLGA FILM

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Human aorta endothelial cells (HAECs) are the layer of flat cells that covers lining the blood vessel. These cells reduce turbulence of the flow of blood, allowing the fluid to be pumped farther. Silk is obtained from the silkworm, it is antioxidant of effects and inhibits blood glucose level. Poly(lactic-co-glycolic acid) (PLGA) is a biodegradable synthetic polymer with acceptable mechanical strength and well-controlled degradation rate. Also, it can be easily fabricated into many shapes. In this study, we fabricated natural/synthetic biomaterial hybrid films using 0, 10, 20, 40, and 80 wt% Silk/PLGA. We performed MTT, SEM, fluorescence staining, ELISA, cGMP assay. We confirmed the adhesion and the proliferation of HAECs on Silk/PLGA according to the content of Silk, the 40 wt% Silk/PLGA films were superior to the adhesion and the proliferation. These results demonstrated that Silk/PLGA films provide suitable surface for HAECs and Silk affects growth and proliferation of the cell. This research was supported by WCU (R31-20029).

Poster Board Number: F-3167

PROLIFERATION AND ERYTHROPOIETIC DIFFERENTIATION OF HUMAN HEMATOPOIETIC STEM CELLS ON CHARGED HYDROGELS

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Stem cells are surrounded by a specialized microenvironment, or 'niche', that ensures their appropriate activities. Especially, hematopoietic stem cells (HSCs) are continuously stimulated by physical interactions with bone marrow or umbilical cord niches and chemical factors from their niches. Interactions between HSCs and extracellular matrix (ECM) in their niche play an important role in proliferation and differentiation of HSCs. Therefore, to clarify the mechanism of the interaction between HSCs and ECM, many studies have been focused on properties of ECM niches, for instance, protein composition, co-culture with stromal cells, substrate elasticity and charge and so on. Recently, it has been reported that positive charged surface with amine group enhanced HSCs' proliferation and terminal differentiation into erythrocytes. In this study, we investigated the proliferation, multipotency and erythropoietic differentiation of HSC on tropoelastin and the hydrogels with positive charge (chitosan, poly-l-lysine) and negative charge (alginate), respectively. According to cell counting, flow cytometric analysis and colony forming assay, it was found that poly-l-lysine significantly increased the cell number in expansion step as well as in erythropoietic differentiation step without changing expression of surface markers related to their stemness. Thus, poly-l-lysine would be a useful substrate to enhance the proliferation and differentiation of HSCs for clinical-grade generation of red blood cells.

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USE OF ROTARY BIOREACTOR SYSTEM TO ENHANCE GROWTH AND OSTEOGENESIS OF HUMAN FETAL MESENCHYMAL STEM CELL

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Bone graft generation *in vitro* is limited by slow nutrients-waste exchange through thick scaffolds and insufficient mechanical stimuli required for induction. Hence, bioreactors were used to provide ample nutrient flow and waste removal through improved fluid flow as well as enhance mechano-transduction to stimulate osteoprogenitor cell proliferation and differentiation. The objective of this study is to determine the ability of modified rotary miniPERM™ bioreactor system in inducing proliferation and osteodifferentiation of human foetal mesenchymal stem cell (hfMSC) on poly-ε-caprolactone (PCL)-tricalcium phosphate (TCP) scaffolds. Briefly, 500,000 hfMSC were seeded onto PCL-TCP scaffolds and cultured for 7 days at rotation speeds of 15 and 20 rpm in modified miniPERM™ with static culture as control. We found that proliferation of hfMSC in 20 rpm dynamic culture was better than its counterparts as evidenced by results from PicoGreen® dsDNA quantitation, light and confocal laser microscopy. Consequently, 20 rpm rotational speed was used to induce and study osteogenesis of hfMSC in the bone induction run. Subsequently, 500,000 hfMSC were seeded onto similar scaffolds and cultured for 28 days in 3 conditions, viz. dynamic induced, dynamic uninduced and static induced cultures. PicoGreen® dsDNA quantitation, light and confocal laser microscopy images revealed the overall growth of cells while ALP activity and calcium content were used to detect osteogenesis. We found that dynamic induced condition in modified miniPERM™ had greater osteoinduction ability with 1.47 times more ALP activity and 3.55 times more calcium deposition as compared to its static induced counterparts. We also noted that a low baseline level of bone induction was seen in the uninduced dynamic culture. This led us to conclude that both osteogenic medium and appropriate mechano-stimulation are critical to effect full osteodifferentiation in hfMSC. The use of rotary modified miniPERM™ bioreactor system improved cellular viability over static cultures, with increased proliferation and osteogenic differentiation *in vitro* suggesting its suitability for bone tissue engineering applications.

Poster Board Number: F-3170

DEVELOPMENT OF MICROCARRIER BASED EXPANSION TECHNIQUE FOR THE BONE TISSUE ENGINEERING APPLICATION OF HUMAN FETAL MESENCHYMAL STEM CELLS

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Despite the self-rejuvenate capacity of bone tissue, large bone defect treatment still remains as a major clinical challenge. Mesenchymal stem cells (MSC) based bone tissue engineering (BTE) strategy has achieved increasing clinical successes, with human fetal MSC (hfMSC) emerging as a superior MSC source for BTE application compared to perinatal and adult MSC sources. In order to achieve

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satisfactory therapeutic efficacy, the cellular expansion approach to propagate adequate number of highly osteogenic MSC (~10^{8-9}) before implantation has become a more pressing need. However, current expansion methodologies based upon monolayer cultures are beset with problems of inefficiency, high cost and decreasing cellular proliferation and osteogenicity caused by the repeated trypsinization treatment. Microcarrier (MC)-based bioreactor culture techniques has been shown to be highly efficient for large scale cell expansion through the large surface area available and the dynamic condition with close monitoring of metabolites and nutrientsmade possible. Hence, in this study, we investigated the potential use of a MC technique for large scale expansion of hfMSC for BTE application. Four microcarriers (Cytodex-1, Cytodex-3, Cultisphere GL and HyQspheres) were screened for hfMSC adhesion and proliferation, with Cytodex-3 being most efficient. Cytodex-3 based MC expanded hfMSC (MC-hfMSC) achieved an expansion efficiency of 12-16 fold and a cell density of 6-8 x 10^5 cells per ml, with the maintenance of high cellular viability (>95%). In contrast, monolayer-expanded hfMSC (ML-hfMSC) only achieved a cell density of 1.2-1.8 x 10^5 cells per ml with 4-6 fold expansion ($p<0.05$, n=5). After harvesting, MC-hfMSC maintain similar cellular characteristics as ML-hfMSC in terms of growth kinetics, CFU-F and immunophenotype. When cultured on the 3D porous scaffold, MC-hfMSC demonstrated increased osteogenic potential, with higher calcium deposition (19.2±0.8 vs. 12.0±0.9 mg, $p<0.05$, n=4), as compared to ML-hfMSC. Furthermore, when implanted subcutaneously into immuno-compromised mice, MC-hfMSC mediated cellular constructs generated more ectopic bone tissue than ML-hfMSC mediated constructs, as shown by micro CT analysis (1.6x, $p<0.05$, n=5) and histological analysis at three months. In conclusion, Cytodex 3 based MC culture technique demonstrated great potential in generating large quantities of hfMSC. Compared to traditional monolayer culture technique, it offers an easy, efficient and scalable approach to propagate hfMSC under highly-controlled conditions, , with significant reduction in the complexity of cell culture procedure, potential risk of contamination, trypsinization treatment and the cell expansion cost. Furthermore, this expansion technique can generate large amount of hfMSC with enhanced osteogenic potential.

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ENHANCEMENTS OF NEO VASCULARIZATION BY TRANSPLANTATION OF HUMAN CORD BLOOD MESENCHYMAL STEM CELLS AS SPHEROIDS.

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Despite promising results from the therapeutic use of stem cells for treating ischemic diseases, poor survival of cells transplanted into ischemic regions is one of the major problems that undermine the efficacy of stem cell therapy. Cord blood mononuclear cells (CBMCs) are an alternative source of mesenchymal stem cells (MSCs) without the disadvantages, such as the painful and invasive harvesting procedure, of MSCs derived from bone marrow or adipose tissue. In the present study, we investigated whether the angiogenic efficacy of CBMSCs can be enhanced by grafting as spheroids in a mouse hindlimb ischemia model. Human CBMSC (hCBMSC) spheroids were prepared by using the hanging drop method. Mouse hindlimb ischemia was induced by excising the femoral artery and its branches. After surgery, animals were divided into no-treatment, dissociated hCBMSC, and spheroid hCBMSC groups (n=8 per group) and received corresponding hCBMSC treatments. After surgery, the ischemic hindlimbs were monitored for 4 weeks, and then the ischemic hindlimb muscles were harvested for histological analysis. Apoptotic signaling, angiogenesis-related signal pathways, and blood vessel formation were investigated in vitro and/or *in vivo*. The transplantation of hCBMSCs as spheroids into mouse ischemic hindlimbs significantly improved the survival of the transplanted cells by suppressing apoptotic signaling while activating anti-apoptotic signaling. Furthermore, the transplantation of hCBMSCs as spheroids significantly increased the number of microvessels and SM ¥á-actin positive vessels in the ischemic limbs of mice, and attenuated limb loss and necrosis. hCBMC can be an alternative source of MSC and spheroid-based CBMSC delivery can be a simple and effective strategy for enhancing the therapeutic efficacy of CBMSCs.

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ENGINEERING A BONE ORGAN WITH HUMAN ADULT MESENCHYMAL STROMAL CELLS THROUGH ENDOCHONDRAL OSSIFICATION

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Adult human Mesenchymal Stromal Cells (hMSC) have typically been used in tissue engineering studies to form and repair bone by a process resembling intramembranous ossification, which relies upon direct differentiation of progenitor cells into osteoblasts. However, most bones develop and repair through endochondral ossification, which consists in transformation of a hypertrophic cartilaginous template into bone. In addition, endochondral ossification presents several advantages for bone regeneration strategies, allowing to overcoming issues critical to the physiological functioning of engineered bone grafts, such as osteogenic performance, resistance to hypoxic environment, vasculogenic potential and, therefore, efficiency of engraftment. In this study, we aimed at developing an upscaled endochondral bone organ model with a "Developmental Engineering" approach by using hMSC. Human MSC were expanded for 2 passages in medium containing FGF-2, seeded onto 8mm diameter, 2 mm thick collagen sponges (UltrafoamTM), cultured for 5 weeks in vitro under chondrogenic and hypertrophic conditions, implanted subcutaneously in nude mice and then retrieved after 5 and 12 weeks *in vivo*. Samples were analyzed by histology (Safranin-o; Alizarin red; Masson's trichrome; TRAP), IHC (Collagen I, -II, -X, bone sialoprotein -BSP-; MMP-9, MMP-13, cleaved aggrecan -DIPEN), biochemistry (Glycosaminoglycans -GAG-; DNA; Calcium), ISH for human Alu sequences and quantitative µCT. Bone marrow was extracted from the samples after *in vivo* culture by mechanical crushing and retrieved cells were characterized by flow cytometry for surface markers phenotype, and cultured in methylcellulose (MethoCult®). In vitro, samples showed a mineralized, pre-bone collar, rich in Collagen I and BSP, and a hypertrophic core, rich in proteoglycans and Collagen X. *In vivo*, extensive remodeling with abundant vessel ingrowth (CD31+ cells) and osteoclast activity (TRAP+ and MMP9+ multinucleated cells) took place. Bone formation displayed a peculiar topography: at the periphery of the samples, perichondral bone was formed, corresponding to the in vitro pre-mineralized outer ring; in the core of the samples, endochondral bone was formed, corresponding to the in vitro non-mineralized cartilaginous areas. Importantly, abundant presence of bone marrow was described. Human cells could be still detected after 12 weeks *in vivo*, mainly in the bone in the core of the samples. Bone marrow was characterized by presence of Lin-, Sca1+, c-Kit+ (LSK) cells, and gave rise to colonies in methylcel-

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lulose culture, suggesting presence of host-derived hematopoietic progenitors within the engineered endochondral bone *in vivo*. Both percentage of LSK cells and number of colonies were comparable to those of the native bone marrow extracted from mice femurs. This work demonstrates that hMSC can regenerate, through endochondral ossification, bone that has features of a mature "bone organ" in an upscaled, ceramic-free scaffold model. Importantly, the engineered tissue was capable to host hematopoietic progenitors, suggesting the formation of a functional bone marrow niche. The findings are relevant towards (i) the development of clinically-sized endochondral grafts capable of enhanced bone repair and (ii) the engineering of ectopic bone marrow niches to study and possibly regulate hematopoiesis in normal and pathologic conditions.

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MATRIX STIFFNESS MODULATES THE DIFFERENTIATION OF NEURAL CREST LIKE-VASCULAR STEM CELLS

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Neural crest like-vascular stem cells (NCL-VSCs) are multipotent and provide a valuable cell source for vascular tissue engineering. However, how extracellular matrix (ECM) regulates the differentiation of NCL-VSCs is not well understood. Here we used collagen and polyacrylamide (PA) gels to mimic the organization of ECM *in vitro*, and determined the effects of matrix stiffness on NCL-VSC differentiation. NCL-VSCs on soft substrates had weaker cell adhesion and lower proliferation rate than the cells on stiff substrates. According to qPCR and immunostaining analysis, NCL-VSCs on stiff substrates induced higher expression levels of smooth muscle cell (SMC) markers myosin heavy chain (MHC), myocardin (MYCD), calponin 1 (CNN1) and smooth muscle α-actin (SMA). In contrast, NCL-VSCs on soft substrates had higher expression of chondrogenic markers aggrecan 1 (AGC1) and collagen-II (COL2A1). Moreover, TGF-β enhanced the expression SMC markers on stiff substrates and of chondrogenic markers on the soft substrates. These results strongly suggest that matrix stiffness differentially regulates stem cell differentiation, and provide significant implications for the design of biomaterials with appropriate mechanical property for tissue regeneration.

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A BIOMIMETIC APPROACH TO SCALE-UP ENGINEERED HUMAN MYOCARDIUM

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Background: Heart muscle can be engineered from primary rodent and also human pluripotent stem cell-derived cardiomyocytes. A potential application of such constructs could be replacement of defective myocardium *in vivo*. Available tissue engineering concepts are limited in scale. Here we took on the biotechnological challenge to generate large human heart muscle pouches with the potential to be tested in larger animal models such as the rabbit. Methods: Based on the anatomical dimensions of a rabbit left ventricle, we reasoned that a tissue engineered ventricular surrogate should accommodate a volume of 4 ml and exhibit a spherical diameter of 20 mm. In addition, we aimed at enabling pulsatile loading. To achieve this, we generated a casting mold (30 mm diameter) containing an inflatable sphere with 20 mm outer diameter localized in its center. An 8 ml reconstitution mixture containing 2.6x107 cardiomyogenically induced HES2 and bovine collagen (6.7 mg) was poured into the mold and allowed to condense for 24 h. From culture days 3 to 8, we initiated uniform pulsatile stretch (15% strain, 90 beats per minute) using a mechanical ventilator to inflate and deflate the central sphere. Non-pulsatile conditions served as control (0% strain). Resulting engineered heart muscle (EHM) pouches were subjected to morphological (confocal microscopy after staining for actinin, actin, nuclei) and molecular analyses (real time PCR) on culture day 8. Results: Stable EHM pouches with a diameter of 23 mm and a wall thickness of 1 mm were obtained on culture day 8. Only pulsed EHM pouches demonstrated clear contractions. Moreover, pulsatile stretch supported the formation of a dense tissue pouch containing elongated cardiomyocytes organized in an anisotropically organized syncytium. In agreement with this morphological observation we found up-regulation of α-cardiac actin mRNA (2.5-fold vs. static control) suggesting advanced sarcomeric maturation. This notion was further substantiated by the observation of an enhanced abundance of transcripts encoding for proteins implicated in mature calcium handling: calsequestrin 2 (CSQ2) - 2-fold; ryanodine receptor 2 (RyR2) - 1.7-fold; sarco/endoplasmic reticulum Ca2+-ATPase (SERCA2a) - 1.2 fold; all compared to static conditions. Conclusion: We demonstrate proof-of-concept for functional myocardial tissue engineering at the rabbit heart scale. This technology is in principle scalable pending on the availability of sufficient cardiomyogenic cell quantities. Whether human sized myocardium can be engineered needs to be investigated.

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FORMATION OF VASCULAR NETWORK STRUCTURES WITHIN THE CARDIAC CELL SHEETS DERIVED FROM MOUSE EMBRYONIC STEM CELLS

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Creation of well-organized vascular network is common issue for the purpose of vital metabolic exchange throughout the engineered 3-dimentional (3-D) tissues. We have developed the cell sheet engineering that makes it possible to create 3-D functional tissues by layering 2-dimentional confluent cell sheets harvested from temperature-responsive culture surfaces and reported that endothelial cell network in neonatal rat cardiac cell sheets enable to connect vessels between host and graft upon *in vivo* transplantation. It has been thought that embryonic stem (ES) cells and induced pluripotent stem (iPS) cells are most possible sources to supply cells in large scale for creating 3-D tissues. Our group aimed to fabricate 3-D functional cardiac tissues derived from pluripotent stem cells and recently reported the creation of cardiac cell sheet by co-culture with cardiomyocytes derived from mouse ES cells and cardiac fibroblasts. For the purpose of reconstructing ES cell-derived 3-D cardiac tissues with vascularization, we present here the formation of vascular network structure in the mouse ES cell-derived cardiac cell sheets co-cultured with CD31+ cells derived from mouse ES cells. ES cell-derived cardiomyocytes were prepared by neomycin selection of differentiated R1 ES cells which expressing EYFP gene ubiquitously and neomycin phosphotransferase gene under the control of the α-myosin heavy chain promoter (R1-neo). To induce differentiation, trypsinized R1-neo ES cells were seeded at 5 × 104 cells/mL into spinner flasks and cultured in the absence of LIF until day 10. For selection of ES cell-derived cardiomyocytes, differentiated R1-neo ES cells were cultured in the presence of G418

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from day 10 to 18. On day 18, undifferentiated cells included in the suspension culture were depleted by magnetic cell sorting (MACS) using anti SSEA-1 antibody. CD31+ cells were prepared from differentiated EMG7 ES cells. To induce differentiation, trypsinized EMG7 ES cells were seeded at 1 × 105 cells/mL into spinner flasks and cultured in the absence of LIF until day 5. On day 5, embryoid bodies were enzymatically dissociated and subjected to MACS to separate Flk1+ cells. Flk1+ cells were re-cultured with both VEGF and 8bromo-cAMP onto ColIV coated tissue culture dishes. 3 days after of the re-culture, induced CD31+ cells were isolated from re-cultured Flk1+ cells by MACS. The expression of endothelial cell markers (vWF, eNOS) was observed in the CD31+ cells. To examine whether the vascular network structure were formed, R1-neo ES cell-derived cardiomyocytes, mouse dermal fibroblasts and EMG7 ES cell-derived CD31+ cells were mixed at ratios of 12:3:1 and seeded at 3.2 × 105 cells/cm2. The formation of CD31+ cell networks was observed after 4days in culture. Total Fiber length calculated from the ES-derived CD31+ cell network structure was the same as that derived from neonatal mouse CD31+ cells. To fabricate cardiac cell sheets with vascular network structure, R1-neo ES cell-derived cardiomyocytes, mouse dermal fibroblast and EMG7 ES cell-derived CD31+ cells were co-cultured onto FBS-coated 24-well temperature responsive culture plate. After 4 days of co-culture, the co-cultured cells were detached as intact cell sheets at 20°C within 1 hour. CD31+ cells network structure was formed within the ES cell-derived cardiac cell sheets. We are now trying to fabricate 3-D cardiac tissues with vascularizaion by layering ES cell-derived cardiac cell sheets with vascular network structure.

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AN IMMORTALIZED HUMAN NEURAL STEM CELL LINE (SPC-01) FOR THE TREATMENT OF SPINAL CORD INJURY

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In the last decade, both embryonic and adult stem cells have been the subject of widespread investigation due to their therapeutic potential in brain and spinal cord injury (SCI). Tremendous efforts have been made to ameliorate and improve locomotor function after SCI by the transplantation of various types of adult as well as embryonic or fetal stem cells. We used an immortalized stem cell line (SPC-01) derived from human fetal spinal cord tissue for the treatment of a balloon-induced spinal cord compression lesion. Prior to *in vivo* experiments, we tested the ability of the cells to differentiate in vitro; specifically, we differentiated SPC-01 cells into motoneurons. To check the functional characteristics of these SPC-01 cells, we measured intracellular Ca2+ concentrations ([Ca2+]i) in individual cells after 14 days of differentiation in culture. We found that 50% of the tested cells (n=24) responded to depolarization by high K+ (50 mM KCl) by a transient increase of ([Ca2+]i) and that these responses were decreased by 50% by the Ca2+ channel blocker nicardipine (n=5), suggesting the presence of high voltage activated Ca2+ channels in this cell population. Furthermore, about 14% of these cells exhibited spontaneous [Ca2+]i oscillations, typically observed in neuronal cells. The [Ca2+]i signals in these cells suggest that SPC-01 cells differentiated into functional motoneurons in vitro. As a model of SCI, a balloon-induced compression lesion at the Th8-9 level was used in adult male Wistar rats. Suspensions of SPC-01 cells (5x105 cells in 5ul of culture medium) were implanted into the lesion one week after SCI (n=22), while the control group (n=16) was injected with saline. SPC-01 cells were labeled with poly-L-lysine-coated superparamagnetic nanoparticles prior to transplantation in order to track the migration and fate of the transplanted cells by MRI. Locomotor (BBB) and sensitivity (plantar) tests were performed weekly for two months. Animals transplanted with SPC-01 cells displayed significantly better motor and sensory improvement compared to the controls. Two months post-implantation (PI), SPC-01 cells robustly survived in the lesion as confirmed by MRI, Prussian Blue staining and staining for the human mitochondrial marker MTC02. Many SPC-01 cells expressed the astrocytic marker GFAP. At two months PI we found 25% of the implanted cells to be positive for Nkx 6.1, and at four months PI the cells were positive for ChAT and Islet2, specific markers for motoneurons. Morphometric evaluation revealed that the white matter was significantly spared in the transplanted rats. Positive staining for GAP43 suggested that SPC-01 cells supported endogenous neurite sprouting and regeneration. Our results demonstrate that the transplantation of SPC-01 cells into the injured rat spinal cord improves functional outcome by partially bridging the spinal cord cavity and by providing trophic support to the spared axons. In chronic lesions, HPMA-RGD hydrogel bridges seeded in vitro with cells were implanted into SCI. The hydrogels filled the post-traumatic cavity, and SPC-01 cells in the hydrogel differentiated into astrocytes and neurons. Host neurofilaments, blood vessels and Schwann cells infiltrated the implant. The combination of SPC-01 cells with biomaterials may be a suitable approach to the treatment of chronic spinal cord injury.

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ADIPOSE TISSUE ENGINEERING IN THREE-DIMENSIONAL LEVITATION TISSUE CULTURE SYSTEM BASED ON MAGNETIC NANOPARTICLES

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White adipose tissue (WAT) is used in regenerative medicine / cell therapy applications, and its physiological and pathological importance is increasingly appreciated. WAT is a complex organ composed of differentiated adipocytes, stromal mesenchymal progenitors known as adipose stem cells (ASC), as well as endothelial vascular cells and infiltrating leukocytes. Two-dimensional (2D) culture that has been typically used for studying adipose cells fails to recapitulate WAT complexity. Improved methods for reconstruction of functional WAT *ex vivo* are instrumental for understanding of physiological interactions between the composing cell populations. Here, we used a three-dimensional (3D) tissue culture system based on magnetic nanoparticle levitation (n3D) to model WAT development and growth in organoids we term "adipospheres". We show that n3D-grown murine 3T3-L1 preadipocytes acquire a hierarchical organization and remain viable in culture for a long period of time, while they lose adherence and die upon reaching confluence in 2D culture. Upon adipogenesis induction, in n3D, 3T3-L1 cells accumulated large unilocular lipid droplets typical of white adipocytes in vivo, while only smaller multilocular lipid droplets accumulated in 2D. Co-culture of 3T3-L1 preadipocytes with murine endothelial bEND.3 cells led to vascular network assembly in n3D and acquisition of a matricellular gradient within the adipospheres. Adipocyte-depleted stromal-vascular fraction (SVF) of WAT cultured in n3D formed organoids with complex hierarchical tissue organization, while growth of SVF in 2D led to loss of endothelial cells and monocytes. Upon adipogenesis induction, n3D-grown organoids derived from the SVF displayed more efficient lipid droplet ac-

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cumulation than 2D cultures, suggesting that adipospheres better recapitulate intercellular signaling required for ASC-dependent WAT organogenesis. Combined, our studies show that the n3D system is advantageous for WAT modeling *ex vivo* and provides a new platform for functional screens to identify molecules bioactive toward individual adipose cell populations. The n3D methodology could be adopted for WAT transplantation applications and aid approaches to WAT-based cell therapy.

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COMPARISON OF THE EFFECT OF SOLUBLE CHONDROITIN-4 AND CHONDROITIN-6 SULFATES ON HUMAN MESENCHYMAL STEM CELL ADHESION, PROLIFERATION AND MIGRATION.

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Human mesenchymal stem cells are a promising cell source for the regeneration of bone and cartilage, which composed mainly of collagens and chondroitin sulfate proteoglycans (CSPGs). It is known that CSPGs participate in the regulation of various cellular activities during the regenerative process. In this study, the effect of soluble chondroitin sulfate (CS), a polysaccharide moiety of CSPGs, on the adhesion, proliferation and migration of immortalized human bone marrow-derived mesenchymal stem cells (hMSC-hTERT-E6/E7) was studied. hMSC-hTERT-E6/E7 were seeded in 24-well tissue culture plates in low glucose Dulbecco's modified Eagle's medium containing chondroitin-4-sulfate (C4S) or chondroitin-6-sulfate (C6S) at concentrations ranging from 0-500 µg/ml. After 4 hr of incubation, cell morphology and adhesion were assessed by light microscope and crystal violet staining assay, respectively. In parallel experiments, 10% (v/v) fetal bovine serum (FBS) was added into the medium after 4 hr of incubation, and the cultures were incubated for further 3 days. Cell proliferation was then estimated in the same way as the adhesion. Soluble CS-mediated migration of hMSC-hTERT-E6/E7 over a 48-hr period was monitored in real-time using the xCELLigence system, whereby migration was quantitated by measuring changes in electrical impedance and reported as an arbitrary Cell Index (CI) value. At 4 hr post-seeding, no apparent morphological differences were observed among C4S-, C6S-treated and untreated (as a control) cells. In controls, nearly 70% of the seeded cells were adhered to the surface. Compared with the control, the addition of 200 and 500 µg/ml C6S to the medium significantly increased cell adhesion by 39 ± 15 and 48 ± 13% ($p < 0.05$), respectively. At low concentrations of 10, 50 and 100 µg/ml, there were no obvious differences from the control. On the other hand, C4S enhanced hMSC-hTERT-E6/E7 adhesion (43 ± 24%; $p < 0.05$) only at the highest concentration. By day 3, significant increases in cell numbers (1.2-1.5 fold) were observed in cultures treated with either C4S or C6S, although they were dose-independent. Furthermore, there was no difference in the cell response between C4S- and C6S-treated groups. In addition, C4S did not exert any distinct effect on hMSC-hTERT-E6/E7 migration, while C6S elicited weak stimulatory effects on the cell migration at concentrations 200 µg/ml. Overall, the highest migration rates of hMSC-hTERT-E6/E7 were observed between 2-6 hr of incubation. The cell migration was time-dependent, with maximal CI values was readily achieved after 12 hr. At this time point, CI values for hMSC-hTERT-E6/E7 in the culture medium with 10% (v/v) FBS (as a positive control) were nearly 2.7 times higher than those treated with 200 or 500 µg/ml of C6S. Our results suggested that soluble C6S and C4S play different roles in regulating hMSC-hTERT-E6/E7 activities. While both polysaccharides exerted positive effects on the hMSC-hTERT-E6/E7 adhesion and proliferation; only C6S had a detectable, though weak, effect on the cell migration.

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FULLY DEDIFFERENTIATED CHONDROCYTE EXPANDED IN SPECIFIC MESENCHYMAL STEM CELL GROWTH MEDIUM WITH FGF-2 OBTAINS STEM CELL PHENOTYPE IN VITRO BUT RETAINS CHONDROCYTE PHENOTYPE *IN VIVO*

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Clinical application of chondrocytes for cartilage repair is very restricted since in vitro expansion of chondrocyte is very limited due to their fibroblastic transition called dedifferentiation. However, previous studies presented that fully dedifferentiated chondrocytes might have characteristics of mesenchymal stem cell (MSC). To evaluate whether dedifferentiated costal chondrocytes (CCs) expressed MSC phenotype in vitro as well as *in vivo*, we cultured CCs in regular chondrocyte growth medium (CGM) or specific MSC growth medium (MSCGM) with or without bFGF up to passage 8, and then the cells were cultured in adipogenic-, osteogenic-, or chondrogenic condition and implanted into animal model. After passage 8, the cells showed fully dedifferentiated phenotype and those dedifferentiated chondrocytes were successfully differentiated into the adipogenic-, osteogenic-, or chondrogenic lineage under in vitro induction conditions. In the ectopic bone marrow formation test, the cells cultured in CGM with bFGF successfully formed bony tissue and ectopic bone marrow. However, the cells cultured in MSCGM with bFGF formed cartilaginous tissue and the tissue was maintained after 16 weeks implantation even in osteogenic condition *in vivo*. Furthermore they restored hyaline cartilaginous tissue on the osteochondral defect in rabbit without calcification events. We conclude that fully dedifferentiated CCs expanded in MSC growth condition with bFGF might be one of optimal cell source for articular cartilage repair, since they retain chondrogenic potential even in osteogenic condition.

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BONE REGENERATION CAPABILITY OF HUMAN BONE MARROW CONCENTRATES VERSUS PLATELET RICH PLASMA

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[Background] Platelet-rich plasma (PRP) containing high concentration of growth factors is well accepted as biological mediators and has been widely used as a treatment approach to facilitate healing process. Bone-marrow aspirate concentrate (BMAC) comprises not only stem cells and progenitor cells in high density but also concentrated platelets. We hypothesized that BMAC might possessed stronger capability for bone engineering, compared with PRP. [Objective] The objective was to evaluate the effects of human BMAC and PRP on promoting initial bone augmentation in mice. [Methods] The BMAC and PRP were concentrated by Megellan automated blood separator from bone-marrow and peripheral-blood aspirates. After cell counting and FACS characterization, specimens

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of three groups (BMAC+TCP, PRP+TCP, only TCP) were implanted to onlay placement on the cranium of nude mice. β-TCP particles were employed as scaffold to carry cells, and samples were harvested after 4 weeks. [Results] After centrifugation, the concentrations of nucleated cells and platelets in BMAC were 2.8±0.8 times and 5.3±2.4 times increased, whereas, leucocytes and platelets in PRP were increased by 4.1±1.8 times and 4.4±1.9 times. The concentrations of CD34, 271, 105, 146-positive cells were highly increased in both BMAC and PRP. Significant new bone area occurred in both sites transplanted with BMAC (7.6±3.9 %) and PRP (7.2±3.8 %) compared to TCP control (2.7±1.4 %). Percentage of new bone in BMAC group was slightly higher than that of PRP group, but the difference was not significant. [Conclusion] These data suggest that both human BMAC and peripheral-blood PRP may provide therapeutic benefits in bone tissue engineering applications, and they possess the similar ability to enhance early-phase bone regeneration.

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CRYOPRESERVATION FOR HEMATOPOIETIC STEM CELLS OF UMBILICAL CORD BLOOD USING CARBOXYLATED E-POLY-L-LYSINE

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Umbilical cord blood (UCB) cells as a source of hematopoietic stem cells (HSC) have been used successfully over the last decade for allogeneic transplantation to treat a variety of genetic, hematologic, and oncologic disorders. Most of UCB cells are stored in liquid nitrogen in individual bags for several years and quickly thawed just prior to transplantation. During cooling, UCB cells are exposed to detrimental effects of ice formation, either directly during rapid cooling and intracellular ice formation or, indirectly during excessively slow cooling, through extracellular ice formation and the damaging effects of the resulting hypertonic solution. To prevent this problem, cryoprotectant agents (CPAs) such as DMSO, glycerol, ethylene glycol, and propylene glycol have been used for the cryopreservation of cells and tissues. The most widely used cryoprotectant is DMSO, but it is toxic and affects the differentiation of many types of stem cells and so needs to be eliminated rapidly after thawing. Many researchers have reported that cryopreservation of stem cells with DMSO, glycerol, or mixtures of CPAs, but few studies focused on the development of novel CPAs. Therefore, we synthesized carboxylated ε-poly-L-lysine (COOH-PLL), an amphoteric macromolecular compound containing both cation and anion (polyampholyte), by chemically modifying polylysine, which is highly safe and frequently used as a food additive. In addition, the best cryoprotective properties were found in a polyampholyte, in which 65 mol% amino groups were converted to carboxylated groups [PLL(0.65)]. We previously reported that COOH-PLL is effective in cryopreservation of many cultured cells such as L929, MG63, HT1680, B16F1, Caco2, MC3T3, and KB cells. In addition, COOH-PLL can also be applied to the vitrification of iPS cells. In this study, we developed an additive to a slow freezing solution comprising ethylene glycol (EG) and sucrose as well as COOH-PLL without DMSO, and compared it with 20% DMSO/2% Dextran and 10% DMSO/12% HES/40% albumin currently used for UCB cells preservation. UCB cells suspensions were cryopreserved in 2 ml cryogenic vials under conditions with -20 °C and -80 °C for at least 2 weeks before thawing and analyzing. Thawed samples were analyzed for viability and absolute CD34+ cell counts by flow cytometry (FACS) and hematopoietic differentiation by using colony-forming unit (CFU) assay. On top of that, GATA-1 was checked for differentiation of megakaryocyte by FACS. In case of the cryopreservation in -20 °C, a solution of 7.5% (w/v) EG, 8.4% (w/v) sucrose and 7.5% (w/v) PLL(0.65) was showed comparatively high viability of a CD34+ cells compared with 20% DMSO/2% Dextran and 10% DMSO/12% HES/40% albumin. In addition, an increase of sucrose would also increase viability of a CD34+ cells. After the cryopreservation at -80 °C of UCB cells with a solution of 10% EG (w/v) and 10% (w/v) PLL(0.65), viability and recovery of a CD34+ cells were significantly higher than those cells with sucrose/PLL(0.65) and 20% DMSO/2% Dextran. Therefore, the use of COOH-PLL is valuable for optimizing cryopreservation agents for HSC from UCB to obtain satisfactory HSC recovery.

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A NOVEL THREE-DIMENSIONAL MODEL OF POSTNATAL *DE NOVO* VASCULOGENESIS FOR ORGAN TISSUE ENGINEERING

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Vascular diseases are one of the leading causes of significant morbidity and mortality worldwide. Vascular diseases not only occur at all levels of vascular tree but also affect multiple organs and organ systems. Organ tissue engineering, including vascular tissue engineering, has been an area of intense investigation. The current major challenge to these approaches has been the inability to vascularize and perfuse in vitro engineered tissue constructs. Attempts to provide oxygen and nutrients to cells contained in biomaterial constructs have met with varying degrees of success. Engineering a tissue of clinically relevant magnitude requires the formation of extensive and stable microvascular networks within the tissue. Since most in vitro engineered tissue constructs do not contain the intricate microvascular structures of native tissue, the cells contained in scaffolds heavily rely on simple diffusion for oxygenation and nutritional delivery. The majority of cells need to be within 100-200 µm of a blood supply to receive adequate oxygen and nutrients for survival. Otherwise, due to diffusion gradients, the cells in the interior regions of the artificial scaffold can experience hypoxia or anoxia and undergo cellular degeneration and necrosis. Hence, this necessitates the formation of appropriate in vitro three-dimensional (3-D) plexuses of new blood vessels within the pre-implanted biomaterial constructs through the process of in situ *de novo* vasculogenesis/angiogenesis for organ tissue engineering. Postnatal bone marrow contains various subpopulations of resident and circulating stem cells (HSCs, BMSCs/MSCs) and progenitor cells (MAPCs, EPCs) that are capable of differentiating into one or more of the cellular components of the vascular bed in vitro as well as contribute to postnatal neovascularization in vivo. When bone marrow stromal cells (BMSCs) were seeded onto a 3-D tubular scaffold engineered from topographically aligned type I collagen fibers and cultured either in vasculogenic or non-vasculogenic media for 7, 14, 21 or 28 days, the maturation and co-differentiation into endothelial and/or smooth muscle cell lineages were observed. Phenotypic induction of these substrate-grown cells was assayed at transcript level by real-time PCR and at protein level by confocal microscopy. In the present study, the observed upregulation of transcripts coding for vascular phenotypic markers is reminiscent of an in vivo expression pattern. Immunolocalization of vasculogenic lineage-associated markers revealed typical expression patterns of vascular endothelial and smooth muscle cells. These endothelial cells exhibited high metabolism of acetylated low-density lipopro-

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tein. In addition to the induced monolayers of endothelial cells, the presence of numerous microvascular capillary-like structures was observed throughout the construct. At the level of scanning electron microscopy, smooth-walled cylindrical tube-like structures with smooth muscle cells and/or pericytes attached to its surface were elucidated. Our 3-D culture system not only induces the maturation and differentiation of BMSCs into vascular cell lineages but also supports microvessel morphogenesis. Thus, this unique in vitro model provides an excellent platform to study the temporal and spatial regulation of postnatal *de novo* vasculogenesis, as well as attack the lingering limit in developing engineered tissues, that is perfusion.

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MIMICKING ENDOCHONDRAL OSSIFICATION IN CUSTOM PERFUSION BIOREACTOR FOR BONE GRAFTS ENGINEERING

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Introduction: It is technologically challenging to engineer bone grafts that possess the physiological properties of the native tissue and this can lead to poor transplant integration. During skeletal development, endochondral ossification takes place during embryonic long bone formation and it is also observed in post-natal fracture healing. Therefore, the purpose of this study was to develop an in-vitro biomimetic bioreactor capable of mimicking the physiological niches essential for endochondral ossification by embryonic stem cells (ESC). Ultimately, achieving bone grafts with anatomical and mechanical properties similar to the native tissues and promote better integration and regenerative capacity. Methods: A custom perfusion bioreactor was built to provide continual media flow to ESC engineered bone constructs at a constant flow rate of 5.5ml/min. The design allows for media modifications in real-time based on the needs of the bone grafts at each stage of differentiation. A collagen 1 platform was fabricated within the bioreactor to support ESC differentiation. Nutrients and oxygen were delivered across an agarose bedding to allow for the generation of gradients within the bioreactor. Results: The custom perfusion bioreactor supported the cultivation of ESCs for at least 52 days. The differential pO2 distribution at the top to bottom of the bioreactor was measured and found to range from 84mmHg at the top to 0mmHg at the bottom. After 52 days in culture, an ivory-toned engineered construct with a rigid texture was derived. The engineered construct was stiffer (144.1Pa) than its cell-free counterpart (21.5Pa). Calcium accumulation throughout the engineered construct was validated through alizarin red straining. Through gene profiling with real-time PCR and subsequent validation with immunofluorescence staining, two major cell populations were found within the engineered construct. These include chondrocytes and osteoclasts, with positional variability observed. The top half of engineered construct exposed to higher level of oxygen was mainly composed of chondrocytes and osteoclasts. In contrast, the bottom half exposed to a hypoxic environment contains only chondrocytes. Although calcium accumulation was detected, results obtained from the gene profile and immunostaining did not indicate the presence of osteoblasts. Conclusion: This bioreactor generates a dynamic environment suitable for extended cultivation, thus enhancing development and maturation of engineered bone grafts. Further, a mineralized construct was derived and is comprised of chondrocytes and osteoclasts. However, the bioreactor requires additional modification for osteoblast differentiation and maintenance. In future experiments, we will apply mechanical stimulants on the constructs to drive further maturation and transplant into critical size skeletal defects. Once validated in vitro and *in vivo*, this technology may provide an alternative approach for producing custom implants containing both bone and cartilage components.

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BIOACTIVE POLY(ETHYLENE GLYCOL) HYDROGEL WELLS SUPPORT THE EXPANSION OF MURINE HEMATOPOIETIC STEM CELL POPULATIONS

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Hematopoietic stem cells (HSCs) have been transplanted for the past fifty years to treat blood diseases and cancers. However, their widespread clinical use has been hindered by the inability to isolate or generate large populations of HSCs. Most HSC expansion techniques focus on the addition of cytokines to culture media or the use of stromal cell feeder layers. There has been limited development of novel biomaterials for HSC expansion. Here, we investigate the use of poly(ethylene glycol) (PEG) hydrogel wells to mimic aspects of the HSC microenvironment, or niche, in an attempt to control or direct HSC expansion. PEG hydrogels resist protein adsorption and subsequent non-specific cell adhesion giving researchers precise control over cell:material interactions. By covalently tethering adhesive peptide sequences and specific proteins to the hydrogel matrix, we can begin to control HSC behavior. In this work, we functionalized PEG hydrogel wells with a fibronectin-derived peptide sequence (RGDS) as well as RGDS in combination with four proteins known to influence HSC behavior: stem cell factor (SCF), stromal derived factor 1α (SDF1α), Jagged1 (JAG1), and interferon-γ (IFNγ). Whole bone marrow was isolated from the long bones of C57 wild type mice and magnetically sorted to obtain a c-kit(+), lineage(-) population. These cells were cultured for 14 days within the bioactive hydrogel wells and subsequently removed to evaluate cell expansion and differentiation potential. The differentiation potential of expanded cells was determined using two methods: flow cytometry and a colony forming unit assay. The functionalization of scaffold surfaces with SCF and IFNγ led to a significant increase in total cell number, 9370% and 10123% respectively, when compared to RGDS alone (4558%), JAG1 (4328%), and SDF1α (4304%). The primitive c-kit(+), Sca1(+), lineage(-) (KSL) population also increased significantly in SCF and IFNγ functionalized wells (660% and 890% respectively) compared to RGDS alone (106%), JAG1 (92%), and SDF1α (312%). Though total cell and KSL expansion on surfaces with covalently immobilized SDF1α and JAG1 was not significant compared to the RGDS only control, the cells maintained their multipotent phenotype as evidenced by a higher frequency of CFU-GEMM and CFU-GM colonies (40% and 43% of total colonies respectively), compared to RGDS alone (26%), SCF (30%), and IFNγ (29%). Our results demonstrate the ability to influence hematopoietic cell expansion and phenotype by tailoring the hydrogel matrix to recapitulate components of the HSC niche. Future work will include further optimization of the system as well as validation of the HSC's functional potential via transplantation studies.

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3D DYNAMIC CELL CULTURE FOR HASC EXPANSION AND MAINTENANCE OF STEMNESS

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Cell has been playing the crucial role in tissue engineering and regenerative medicine. It has been more than a decade since the scientists started putting endeavors into stem cell researches and great milestones of tissue engineering approaches have been achieved. However, the finite amount of cells that can be extracted from an individual remains an impediment in doing experiment. Monolayer cell expansion on cultivating plate is a general way to proliferate cells. Though it is easy to operate, it has the problem of maintaining the phenotype and causing the loss in cell-extracellular (ECM) interactions by proteolytic enzyme treatment. To improve cell culture condition, 3D cell culture which increase cell viabilities and growth rate by culturing cells in environment similar to real body and reduce harm of cell ECM at the same time is used. Besides, microcarrier which is involved in 3D culture has the advantage of high surface to volume ratio, efficient oxygen and medium nutrient diffusion and maintaining cell phenotype. In this study, we used hASC (human adipose-derived stem cell) as our model to compare the cell expansion rate and differential ability in 2D and 3D cell culture. hASC secretes growth factors which induces angiogenesis and is commonly used in regeneration of ischemia tissue, but has the disadvantage of limited cell sources and hard to maintain in cell culture due to slow growth rate. GEM (magnetic Global Eukaryotic Microcarrier) and Cytodex3 were used as the microcarriers for 3D cell culture. GEM is composed of an alginate core embedded with paramagnetic particles and coated with covalently bound adhesion molecules, such as, collagen-I, collagen-IV, gelatin and laminin. Cytodex3 are transparent microcarriers which consist of a thin layer of denatured collagen chemically coupled to a matrix of cross-linked dextran. We performed the experiment from cell adhesion assay with different coating GEM and find the most proper coating for hASC then compare rate of cell growth in 2D or 3D culture. BioLevitator, a bench-top incubator which can increase the mass transfer between cells and microcarriers by tuning the rotating condition to thoroughly mixing is used as the 3D culture system. Cells morphology and proliferate state is observed by DAPI and Phalloidin staining with fluorescent microscopy. Cells from either 2D or 3D culture are replated in 24-well plate to test their differential ability toward osteogenic and adipogenic lineage. The ultimate goal of the study is to obtain sufficient cell numbers which also have higher viabilities and differential abilities in short period to help with further experiment in tissue engineering.

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EFFECTS OF HESPERIDIN/PLGA FILMS ON GROWTH AND PHENOTYPE OF RETINA PIGMENT EPITHELIAL CELLS

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Transplantation of retinal pigment epithelium (RPE) has recently become a possible therapeutic approach for retinal degeneration. Allograft tissue replacement is a relatively high host refection rate. One of the scaffolds, Hesperidin, it is currently proved to be biological safe. We fabricated PLGA film as a control group and Hesperidin/PLGA films as an experiment group. RPE cells were seeded on Hesperidin/PLGA films, and we studied the SEM, WST, Histology and RT-PCR. In this study, we confirmed that the cell viability was higher percent of Hesperidin/PLGA films through WST. The WST results, cell proliferation was good in higher percent of Hesperidin/PLGA films. The RT-PCR results showed specific mRNA expression. In conclusion, this study suggested that Hesperidin/PLGA film may serve as potential RPE cells differentiation and structural basis for tissue engineered RPE cells. This research was supported by WCU (R31-20029), and SCRC (SC4110).

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CD34+ EPITHELIAL STEM CELLS COULD BE USED WITH DECELLULARIZED SCAFFOLDS FOR OESOPHAGEAL TISSUE ENGINEERING

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Aim: Replacement of the esophagus for pathologies such as long-gap oesophageal remains a challenge to surgeons and is associated with significant morbidity. Tissue engineering may provide a safer alternative to current techniques. The aim of this study was to: (i) isolate oesophageal epithelial stem cells, (ii) compare two techniques of oesophageal decellularization with the view of combining the two and creating a construct Methods: Following ethical approval, the oesophagus was resected from mice and incubated in dispase, following which mucosa was separated from the submucosa. Epithelial cells (mucosa) and fibroblasts (submucosa) were isolated using trypsin/EDTA and dispase/collagenase incubations respectively. Oesophageal epithelial cells were either plated or stained with anti-CD34 and sorted using FACS. Data are expressed as median (range). The sorted CD34+ epithelial population was seeded on mitotically inactivated 3T3 fibroblast feeder cells. In parallel, oesophagus was isolated from rats (n=10) and decellularized using two methods: (a) detergent-enzymatic treatment (DET) comprised of deionized water, sodium deoxycholate, and DNase-I; (b) 1% sodium dodecyl sulphate (SDS). Samples were analysed using DNA and collagen quantification, Haematoxylin and Eosin (H&E), Masson's Trichrome, Elastin Van Gieson and DAPI staining. Results: Epithelial cells and fibroblasts were successfully isolated from mouse esophagi. Using FACS, CD34low 5.7% (3.8-7.6) and CD-34high 0.5% (0.2-0.8) positive populations were detected (Fig.1a) and successfully formed colonies in culture when seeded on feeder layers (Fig 1b). Colonies had a cobblestone morphology similar to that present in epithelial cells that were plated without FACS selection. Regarding the scaffold production, H&E and DAPI staining demonstrated no cellular remnants following 1 cycle of DET and 6 hours of SDS respectively. However, histological results show clear loss of microarchitecture in the SDS-treated oesophagus with a poor connective tissue network. Elastic fibers are significantly more disrupted in the SDS compared to the DET-treated oesophagus. Conclusion: CD34+ epithelial stem cells and fibroblasts can be successfully derived from mouse oesophagus. In rodents, the best course of decellularization is the DET as it causes the least disruption of the structure and components of the extracellular matrix whilst still removing all cellular remnants. The combination of decellularised oesophagus, CD34+ cells and fibroblasts could advance the development of a viable tissue engineered neo-oesophagus.

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STRATEGY TO ENHANCE THE EFFICIENCY OF ENDOTHELIAL CELLS-BASED THERAPY IN PHYSIOLOGICAL AND PATHOLOGICAL WOUND HEALING

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We aimed to analyze the effect of endothelial progenitor cells (EPC) on wound healing process in physiological and pathological conditions. We have also developed novel approaches based on pre treatment with ephrin B2-Fc or co administration with smooth muscle progenitors cells (SMPC) to improve the therapeutic efficiency of EPC -based strategies. At Day 3, EPCs promoted dermal wound healing and enhanced wound closure in both non irradiated and irradiated mice by 2.1- and 2-fold respectively compared to PBS injected animals. At day 7, EPCs improved skin blood perfusion and capillary density in both irradiated and non irradiated mice. Of interest activation of EPC with an ephrin-B2-Fc (Eph B2) increased the wound closure by 1.6 -fold compared to unstimulated EPC in non-irradiated mice. However, the beneficial effect of EphB2 is abolished in irradiated animals. Likely due to the overproduction of reactive oxygen species and inhibition of EPC adhesion. In irradiated condition, NAC treatment restored the beneficial effect of Eph-B2-Fc treated EPC on wound healing and promoted adhesion to endothelial cells as assessed by real time video microscopy. Finally, co therapy strategy using both EPC and SMPC co administration improved wound closure after irradiation. In conclusion, we showed that Eph-B2-Fc pre-treatment improved the beneficial effect of EPC in physiological conditions and irradiated conditions with antioxidant treatment. Co therapy of EPC and SMPC has proved beneficial effect in pathological conditions. All together our study open the way for clinical development of efficient EPC-based strategies in wound healing.

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IMPORTANCE OF PRESERVING ECM FOR THE RECONSTRUCTION OF HEMATOPOIETIC STEM CELL NICHE

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Introduction: Hematopoietic stem cells (HSCs) have the ability to self-renew and the ability to produce multiple lineages of daughter cells. Hematopoietic stem and progenitor cells (HSPCs) are thought to reside in specific niches that are specialized microenvironments within the bone marrow cavity. In the hematopoietic stem cell niche, the proliferation and differentiation of HSCs are regulated by biological substances secreted from various supporting cells. Furthermore, it is also reported that the role of extracellular matrix (ECM) is an important factor which alters the fate of the cells, but its role is not fully understood. We hypothesized that by preparing a matrix which possesses the exactly the same structure to that to the bone marrow and adding the supporting cells, it would be possible to regenerate the hematopoietic function of the bone marrow and define the role of the ECM much specifically contemporarily. For the matrix, we chose decellularized bone marrow (DBM). This is because the decellularization eliminates the cells but the structure of the ECM remains. So, by adding mesenchymal stem cells (MSCs), which are one of the supporting cells, to the DBM, it is expected that the 3-D structure template of the artificial niche can be provided. In this study, we report on the importance of the preserving ECM for the reconstruction of the hematopoietic stem cell niche. Materials and Methods: The porcine costae were used as a matrix for the experiment. The decellularization was executed by detergent treatment or by high pressure treatment. For detergents treatment, the costae were treated with SDS or TritonX-100 solution. For high pressure, high-hydrostatic pressure (HHP) method was used. HHP method was executed by pressurization the samples at 980 MPa to disrupt the cells, followed by washing the samples to remove the residues of the disrupted cells. The decellularization was evaluated by histological assessment and the residual DNA quantitation. Then, the samples with lower than 1 % of residual DNA were chosen for human MSCs (hMSCs) culturing. Alkaline phosphatase (ALP) staining was performed to evaluate the cell adhesion within the scaffold. In order to establish the relationship between hMSCs and hematopoiesis, DBM seeded with hMSCs was implanted to mice subcutaneously. After 2 weeks, the sample were taken out and subjected to H-E staining. Results and Discussion: For SDS and HHP method, the residual DNA in decellularized bone decreased to approximately 1 % of native bone. TritonX-100 solution could not eliminate the cells completely. The reticular tissue and lipid droplet remained within the bone marrow when HHP or tritonX-100 method was used. This implies that the HHP is the only method for complete cell elimination while maintaining the original bone marrow structure including lipid droplet. The existence of reticular tissue was important when culturing hMSCs, for the adhesion of the cells within the bone marrow was promoted. When the DBM recellularized with hMSC was implanted subcutaneously, we found out that the neovascularization was promoted compared to that to the DBM culturing no cells. Furthermore, the red blood cells outside new blood vessels were observed, implying the homing of mouse HSPC. Conclusion: Our study revealed that the maintaining of the original structure of bone marrow and the recellularization of hMSC is very important the reconstruction of the hematopoietic stem cell niche, because this promotes the hematopoietic function in the living body.

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PREPARATION OF PHOSPHATASE-RESPONSIBLE HYDROGELS CAPABLE FOR THE STIFFNESS CHANGE ASSOCIATED WITH MESENCHYMAL STEM CELL DIFFERENTIATION

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Recently, the stiffness of cell substrates has attracted much attention in biological researches as a physical signal to regulate the motility, proliferation, and differentiation of cells. It is demonstrated that the substrate stiffness plays a crucial role in directing cell behaviors, such as the lineage specification of stem cells, the motility of tumor cells, and the *in vitro* epithelial-mesenchymal transition. The objective of this study is to design stimuli-responsive hydrogels, the stiffness of which can be changed by phosphatase of an extrinsic cues in cell culture. Mesenchymal stem cells (MSC) were cultured on the phosphatase-responsive hydrogel in the presence or absence of phosphatase. The morphology of MSC was observed by microscopy while their osteogenic differentiation was evaluated. Acrylamide, N,N'-methylenebisacrylamide (BIS), and methacryloyloxyethyl phosphate of Phosmer M (Uni-Chemical co., Ltd) were

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co-polymerized to prepare hydrogels containing phosphate ester groups susceptible to enzymatic dissociation by alkaline phosphatase (ALP) of an osteogenic marker. The stiffness of hydrogels changed by their exposure to ALP. The magnitude of stiffness change by ALP treatment increased with an increase in the concentrations of either BIS or phosphate ester groups. When placed in the culture media containing ALP, the hydrogel became softer by the enzymatic dissociation of phosphate ester groups.To investigate the effect of stiffness change on the osteoblastic differentiation of MSC, the cells were cultured on the phosphatase-responsive hydrogels with different magnitudes of stiffness change. Following the hydrogel was treated with ALP to become softer, the morphology of MSC became more spindle on the hydrogel several days after culture. When evaluated by quantitative polymerase chain reaction, the expression level of RUNX2 of an osteogenic marker was increased changed by changing the magnitude of hydrogel stiffness. These results indicate that the MSC fate was influenced by changing the stiffness of cell substrates during culture.

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NOVEL APPROACH TO MICROENCAPSULATE MESENCHYMAL STEM CELLS TO INHIBIT IMMUNE RESPONSE AND REPAIR SKELETAL DEFECTS IN PATIENTS WITH CANCER INDUCED BONE DISEASE

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Metastatic cancer in Women is not well understood and little has been developed to address or minimize the damage caused by the growing tumor cells in bone tissues. High doses of radiation combined with chemotherapy are often used to eradicate all the metastatic tumor cells; however the end result for many women is osteoporosis and bone fragility. In addition, surgeons are gaining expertise in removing metastatic tumors from the spine and other hard tissues; however bone regeneration is slow and very painful for these patients. An approach to encapsulate mesenchymal stem cells secreting osteogenic growth factors has been developed as a tool for bone tissue regeneration studies in rat/mouse models. MSCs have demonstrated success in repairing non-healing bone defects in humans, but obtaining enough host MSCs requires harvesting and expanding the cells, a process which takes several months. Our hypothesis is that microencapsulation will protect the MSCs from immune attack and will permit the engraftment and recruitment of endogenous stem cells in human tissue repair models. If this method is successful, the immortalized encapsulated MSCs can be an immediate "Off the shelf" therapeutic for a variety of human tissue repair systems (e.g. bone, heart, liver, dental' etc.). In this abstract, we utilize the human fetal mesenchymal stem cell (MSC) line isolated, characterized and immortalized in by expression of telomerase reverse transcriptase (hTERT; the modified cells are denoted as hTERT-MSC). hTERT-MSC demonstrate the ability to differentiate into all the mesenchymal lineages including osteoblasts. In collaboration with Dr. Jacob Berlin, the hTERT-MSCs are microencapsulated and introduced into three type of bone repair systems (Spinal fusion, craniofacial, and endochondral bone) to induce bone formation in athymic rats. • Heterotopic Bone Formation Study - This part of the study will be used as a pilot study to determine whether the microencapsulated MSC cells are capable of inducing heterotopic bone formation in NSG mice by intramuscular injection and monitoring heterotopic bone formation. Bone formation is analyzed by x-ray, micro-computed tomography (microCT) and histology after euthanasia. • Critical Size Defect Studies - Following confirmation of bone formation in the heterotopic study, the microencapsulated MSC cells are delivered to surgically produced critical size bone defects: calvarial, fibular and femoral repair models in athymic rats. Repair is evaluated by x-ray, microCT, and following euthanasia biomechanical and histological analysis will be performed. • Spinal Fusion Study - Microencapsulated MSC cells delivered to the spine elicit spinal fusion. Evaluation of spinal fusion and vertebral bone quality are assessed by x-ray, microCT, and biomechanical and histological analysis. Results of the above studies will be discussed in the session.

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FABRICATION OF MESENCHYMAL STEM CELL AGGREGATES CONTAINING SUGAR-RESPONSIVE GELATIN HYDROGEL MICROSPHERES INCORPORATING BONE MORPHOGENETIC PROTEIN-2

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Cell-cell interactions are essential to regulate cell functions in the living system. To realize the cell-cell interaction *in vitro*, cell aggregates with a living tissue-like structure of 3-dimension has been extensively investigated. However, when the size of cell aggregate becomes too large, cells inside the aggregate tend to die due to their lack of oxygen and nutrients. To overcome this drawback, we have developed a culture technique by making use of gelatin hydrogel microspheres and demonstrated significance of the technique in improving the cell survival in mesenchymal stem cells (MSC) aggregates. The incorporation of hydrogel microspheres promoted glucose methabolism in the MSC aggregate. In this study, as a new system, we design a hydrogel microsphere which not only functions initially as a cell scaffold, but also can be removed without any cytotoxicity when the microsphere dose not need anymore. As such a substrate, sugar-responsive gelatin hydrogel microspheres incorporating bone morphogenetic protein (BMP)-2 were prepared. Briefly, m-aminophenylboronic acid (APBA) of a sugar-responsive moiety was introduced into gelatin with a weight average molecular weight of 100,000 and an isoelectric point of 5.0 (Nitta Gelatin Inc., Japan) by using N-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. An aqueous mixture of the APBA-introduced gelatin and poly(vinyl alcohol) (PVA) was emulsified in olive oil at 60 oC and solidified by cold acetone to obtain hydrogel microspheres. The resulting hydrogel microspheres were air-dried and sterilized with ethyleneoxide gas. Then, an aqueous solution of BMP-2 was impregnated into the air-dried microspheres. When placed in a culture media with sorbitol of a sugar, BMP-2 was released from the BMP-2-incorporating hydrogel microspheres by their water-solubilization. However, in the absence of sorbitol, the hydrogel microspheres were stable and no BMP-2 release was observed. These findings indicate that sorbitol disrupts the crosslinking of hydrogel microspheres, leading to their water-solubilization and the subsequent BMP-2 release. MSC were isolated from the bone marrow of rat femora and tibiae, and co-cultured with the hydrogel microspheres incorporating BMP-2 in 96-well tissue culture plate coated with an agarose gel under horizontal rotation. After co-culture with MSC for 7 days, stable cell aggregates containing the hydrogel microspheres were formed. Upon adding sorbitol in the culture media, the size of cell aggregates rapidly increased by about 30 % and gradually decreased to the original size within 48 hr. During the process, the hydrogel microspheres

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disappeared in the cell aggregate. No cytotoxicity was observed after the sorbitol addition. It is concluded that the present hydrogel microsphere system of sugar-responsive water-solubilization is a promising culture substrate to allow cells to form a living tissue-like structure of 3-dimension.

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MOLECULAR ANALYSIS OF CULTURED ORAL MUCOSAL EPITHELIAL CELL SHEET FOR CORNEAL REGENERATION

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Eye diseases and ocular trauma cause defect in corneal regeneration which leads to corneal opacification and then blindness. Autologous stem cell transplantation for such defects is immunologically preferable to avoid allograft rejection. In the present studies, novel tissue engineering approaches for ocular surface reconstruction are gaining a keen interest and achieving significant outcomes. Among them, it has been reported that, advanced with a unique culture technique on temperature-responsive polymer-covered cell culture surfaces, Cell Sheet Engineering enables the production of intact (maintaining cell-cell junction and basal membrane components), highly organized, and stem cell-containing transplantable graft (cell sheet) that leads to the excellent delivery and subsequent favorable efficacy and stability. Our current research aim is to exploit the molecular characteristics of the engineered graft to answer to the ultimate topic that how the stem cell-containing graft without any artificial scaffolds can precisely be described in the molecular language, elucidating the reasons for the successful outcomes. To address this, we carried out histological and biochemical analyses of rabbit oral mucosal epithelial cell sheets cultured on and harvested from the temperature-responsive cell culture surfaces, focusing on several significant molecules relevant to the epithelial cell system. H&E staining of the cell sheets demonstrated a stratified epithelium with 4-6 cell layers, suggesting a reconstruction of the normal epithelial cell system even on the polymer-covered surface. While the apical side of the cell sheets stained positive by the immunofluorescent (IF) staining with CK4, a molecular marker of the surface layer of the stratified epithelium, IF analyses with both the stem cell markers, Integrin beta-1 and p63, were identified in the basal layer of the stratified epithelium. These results indicate the asymmetric localizations of stem/progenitor cells in the basal layer as well as differentiated cells in the apical layer, consistent with the previous reports on epitheliums. Moving forwardly, we conducted a biochemical analysis focusing on several cell stress-related molecules.

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STRUCTURE FORMATION OF TISSUE-ENGINEERED EPITHELIAL PROGENITOR CELLS OF GLANDULAR ORGANS

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Glandular organs are important in regulating enzymes, metabolites, and fluid transport to maintain physiological homeostasis. The branching structure is the characteristic tissue feature among many different glandular organs, and by which different types of cells can be organized to efficiently fulfill physiological functions. To regenerate functional glandular organs, recapitulation of tissue-specific structure formation is required. The aim of this study is to develop a biocompatible system that can generate branching structures of glandular organs by engineering epithelial progenitor cells without exogenous supplement of serum or growth factors. The fetal mammary gland (MG) and the submandibular gland (SMG), which are typical glandular organs responsible for synthesis and secretion of milk and saliva, were selected as the model organs for investigation. Because of the advantages of a serum-free system for clinical translation, the cells were engineered with biocompatible biomaterials instead of exogenous supplement of serum or growth factors. Among the many biomaterials used to engineer epithelial progenitor cells, chitosan demonstrated a superior effect in promoting MG and SMG branch formation. The morphogenetic effect toward explant branching was chitosan specific and not observed in other analogues with similar chemical compositions or structures. The molecular weight and specific linkages in the chitosan polymer were important parameters in mediating the morphogenetic effect. MG explants from different anatomical locations effectively promoted structure formation. Likewise, SMG explants demonstrated similar effects when the progenitor cells were engineered in the chitosan-containing system. These results indicate the effect of chitosan-containing system is versatile in facilitating structure formation of different glandular organs. In MG explants, blocking endogenous FGF10 inhibited the morphogenetic effect of chitosan, indicating that the chitosan effect was FGF10 dependent. This work demonstrates the feasibility of creating a bioreactor system free from exogenous growth factors and serum that is competent to facilitate tissue structure formation by engineering epithelial progenitor cells. The process of tissue structure formation of glandular organs can be efficiently recapitulated and promoted in a biocompatible manner that is beneficial to clinical translation.

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ENDOTHELIAL PROGENITOR CELLS EXPANDED *IN VITRO* ARE MORE EFFICIENT FOR THE TREATMENT OF RATS WITH INFARCTED MYOCARDIAL.

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Introduction and Objectives: Endothelial progenitor cells (EPCs) have proven to be efficient in the process of neovascularization of ischemic areas. The aim of this study is to compare the therapeutic potential of purified and expanded umbilical cord blood (HUCB)-derived EPCs by analyzing the results of echocardiography (ECHO) and histology of infarcted hearts 30 days after transplantation. Methods: EPCs were selected using CD133-coupled magnetic microbeads. The cells were expanded *in vitro* for 30 days in media supplemented with growth factors b-FGF, VEGF and IGF-I and fetal calf serum. A model of myocardial infarction (MI) in rats was established by ligation of the anterior descending coronary artery. Only rats with ejection fraction (EF) below 40% remained in the study. The animals were divided into three groups with 10 individuals each. Group A received purified EPCs, the group B received expanded EPCs and the group C (control) received only saline buffer. The transplants were performed on the ninth day after MI; and 2x105 cells were injected intramyocardially in the central infarction area. After four weeks the left ventricular function was assessed by ECHO, the number of capillaries was counted in slides stained with HE and the presence of transplanted cells was evidenced by FISH. Results: The variation of EF results by groups was: B 28.6% to 40% with a significant p = 0.006, A 31.1% to 38.3% with a strong trend of improvement p = 0.06 and C 27.4% to 29.9% with no statistical

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difference p = 0.429. The capillary count was higher in groups A and B compared to C but with no statistical difference among them all. It was found capillaries of immature and primordial aspect in group A and, more mature capillaries with wide vascular connection in group B. Donor cells were founded in the infarcted area of all animals in groups A and B. Conclusion: HUCB-derived EPCs were able to be incorporated into the infarcted myocardial tissue; they induce neovascularization and improve left ventricular function. The group of expanded EPCs preserved the systole, prevented dilation and remodeling, also recovered the EF. Although increased capillary density was similar among the group cells group B was the one with most developed aspect. It is therefore suggested that expanded EPCs could be a better therapeutic option in the future.

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DIFFERENTIATION ABILITY AND PLURIPOTENCY OF AMNIOTIC FLUID DERIVED STEM CELLS CULTURED ON EXTRACELLULAR MATRIX IMMOBILIZED SURFACE

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Mesenchymal stem cells (MSCs) are one of the most widely available autologous sources of stem cells for clinical applications. Stem cells derived from amniotic fluid are pluripotent fetal cells capable of differentiating into multiple lineages, including representatives of the three embryonic germ layers. Therefore, amniotic fluid may become a more suitable source of stem cells in regenerative medicine and tissue engineering than embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) due to the lack of ethical concerns regarding use of ESCs and the lack of concerns about xenogenic contamination arising from the use of mouse embryonic fibroblasts as a feeder layer for iPSCs. However, stem cell characteristics, such as proper differentiation and maintenance of pluripotency, are regulated not only by the stem cells themselves but also by their microenvironment. Therefore, mimicking stem cell microenvironments using natural biomacromolecules, such as extracellular matrix (ECM) proteins, facilitates the *in vitro* production of the large numbers of pluripotent stem cells and specifically differentiated cells needed for regenerative medicine In this study, stem cells from amniotic fluid were cultured for several passages on dishes grafted with extracellular matrix (ECM) or Matrigel where gelatin, collagen, fibronectin, laminin, and vitronectin were selected as ECM components (nanosegments). The effects of interactions between amniotic fluid stem cells and nanosegments were investigated on the expression of pluripotent genes (e.g., Oct4 and Nanog) and on the differentiation abilities of osteoblasts at each passage. The ECM-grafted dishes produced water contact angles from 40 to 65 degrees, which was an adequate water contact angle range for the cell culture. Culture on ECM-immobilized dishes enhances amniotic fluid stem cell differentiation into osteoblasts more than culture on polystyrene dishes grafted with amino groups (PS-NH2 dishes). This finding indicates that specific interactions between amniotic fluid cells and the ECM grafted onto the culture dishes promote the differentiation of cells into osteoblasts. Immobilization of the optimal nanosegments (ECM or Matrigel) onto culture dishes enhances amniotic fluid stem cell differentiation into osteoblasts; the choice of nanosegments depends on the desired differentiated cell type. We will discuss the optimal ECM-grafted dishes, which keep pluripotency of the amniotic fluid stem cells for a long time (i.e., at late passages).

Totipotent/Early Embryo Cells

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AUTO- AND CROSS-REGULATION OF CDX4 AND SALL4 REGULATES THE TRANSITION FROM MESODERM FORMATION TO EMBRYONIC HEMATOPOIESIS

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Hematopoietic stem cells (HSCs) arise from mesoderm following gastrulation. Previous studies on zebrafish with a mutation in the caudal transcription factor cdx4 demonstrated a prominent defect in the ability of mesoderm to become blood. Here, to understand how cdx4 contributes to embryonic hematopoiesis, we determined the direct binding sites of Cdx4 in zebrafish embryos using ChIP-seq technology, examined gene expression data in the cdx4 mutants using microarrays, and evaluated a critical genetic interaction between cdx4 and one of its targets, sall4. ChIP-seq analysis showed Cdx4 binding to the hox genes, the Wnt pathway genes, and the BMP pathway genes. In addition, cdx4 gene itself and the spalt-like 4 (sall4) transcription factor gene, a member of the embryonic stem cell network, is transcriptionally regulated by Cdx4. Sall4 ChIP-seq reveals that Sall4 binds to its own promoter and to the cdx4 promoter, suggesting an auto- and cross-regulation between Cdx4 and Sall4. A comparison between the Cdx4 and Sall4 bound genes shows that both transcription factors bind to mesodermal progenitor genes (including spt (tbx16), and mespa) and hematopoietic-specific transcription factor genes (scl, gata2a, and ldb1a). Based on this data, we hypothesized that Cdx4 and Sall4 directly affect the mesoderm to blood transition by co-regulating a set of genes. In an effort to examine genetic interactions between Cdx4 and Sall4, sall4 was knocked down in the cdx4+/+, cdx4+/-, and cdx4-/- background by morpholino (mo). sall4 knockdown alone did not cause hematopoietic defects in zebrafish embryos, yet its knockdown in the cdx4-/- background led to a severe decrease in red blood cells (RBCs) as shown by *in situ* hybridization (ISH) for gata1 mRNA (17/19) and o-dianisidine staining (6/8). As the loss of RBCs could be caused by earlier defects during gastrulation, other mesodermal tissue genes notail and eve1 (even-skipped 1) were examined by ISH alongside the hematopoietic specific genes scl and lmo2. scl (6/6) and lmo2 expression (5/5) in the posterior tissue was absent in the sall4mo;cdx4-/- embryos, yet notail (10/12) and eve1 (6/6) expression was minimally affected, indicating that cdx4 and sall4 co-regulate mesoderm specification to blood. Taken together, we conclude that auto- and cross- regulation of Cdx4 and Sall4 during gastrulation results in a stable molecular circuit that plays a critical role in the commitment of mesoderm to the blood lineage.

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FUNCTIONS OF TRANSCRIPTION FACTORS IN SOMATIC PLURIPOTENT STEM CELLS OF PLANARIANS

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The freshwater planarian *Dugesia japonica* has somatic pluripotent stem cells called neoblasts in the adult body, in which the neoblasts can give rise to all types of cells, including germ cells. The neoblasts are localized in the mesenchymal space throghout the body, and constitute about 30% of the total cells in planarians. Planarians possess extremely high regenerative capability, by which tiny fragments of alomost any body region can regenerate and develop into complete functional individuals within one week. This ability is dependent on the neoblasts. During the regeneration process, the neoblasts supply all types of cells required for rebuilding missing tissues or organs, such as eyes, brain and pharynx. Recently, it has been reported that many genes encoding RNA-binding proteins, especially germline-specific RNA-binding proteins, are specifically expressed in the neoblasts, and play role in various stem cell functions, such as cell maintenance, proliferation and differentiation. When the gene function of those genes is inhibited by RNAi, planarians totally lose their regenerative ability because of the resuting defects in all of the neoblasts. Because of this problem, cell-type-specific differentiation of the neoblasts is still poorly understood. It is well known that transcription factors are involved in several different aspects of the regulation of stem cells, including maintenance of pluripotency, proliferation, and specific differentiation. Therefore, to dissect these different aspect, we focused on transcription factors expressed in the planarian neoblasts. Our FACS-based single cell PCR method revealed that at least 8 genes encoding independent transcription factors were mutually exclusively expressed in the population of neoblasts. Gene knock-down experiments by RNAi clarified that HLH-1, a planarian helix-loop-helix type transcription factor, and FoxA were required for regeneration of the pharynx and muscle-surrounding cells, which are located in the central portion of the body. HLH-1 was also involved in the differentiation of mucus-producing cells from the neoblasts. In this mucus-producing cell differentiation, we found that NatoA and Dth-2, a homeobox gene, also functioned in downstream of HLH-1. Two differentiation pathways of the neoblasts begining at HLH-1 showed different requirements for ERK signaling: the expression of FoxA followed by pharynx regeneration and muscle-surrounding cells differentiation required a low level of ERK signaling, but the differentiation of mucus-producing cells did not require it. In order to define the molecular basis of the neoblast cell state, we performed systematic gene expression analysis by HiCEP and thereby identified several transcription factors expressed in the neoblasts. Among them, we found that p53 and a Sox homolog, named SoxP1, regulated the production of a certain population of cells, which we called Type1 cells, whose fuction is still unclear. Thus differentiation of the neoblasts seems likely to be strictly regulated by combinatory pathways of various transcription factors. Here, we would like to discuss about the mechanisms of the regulation of neoblasts by transcription factors in planarians.

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PATTERN OF EARLY GENE EXPRESSION DURING EARLY MARMOSET DEVELOPMENT

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Mouse embryonic stem (ES) cells are pluripotent and can produce chimeras with blastocyst injection. Many gene functions have been revealed by chimeric mice that have been produced by targeted gene knockout mouse ES cells. Primate ES cells are also pluripotent, but lack the ability to produce chimeras. Although ES cells are derived from the blastocyst inner cell mass (ICM) in mice and primates, morphologically the primate ES cells resemble the pluripotent cell lines derived from post-implantation mouse epiblasts. It has been postulated that there are differences in the developmental mechanisms of mice and primates. To clarify these differences, we performed immunostaining to analyze the expression of the Oct3/4, Nanog, Sox2, and Gata4 genes during early development in the common marmoset (Callithrix jacchus). We found that the pattern of early gene expression in the marmoset differed from that reported in the mouse. Oct3/4 expression was detected in the nuclei of 8-cell stage embryos. In addition, Oct3/4 was co-expressed with Gata4 in each blastomere until the morula. In the blastocyst stage, the co-expression of Oct3/4 and Gata4 could be detected throughout the embryos, including the ICM and trophectoderm (TE). The expression pattern of Oct3/4 and Gata4 was clearly separated in hatched late blastocysts. Oct3/4 expressed in the ICM was surrounded by Gata4 expression, indicating that specific signal transduction controls the gene expression from the blastocyst to the hatched late blastocyst. This developmental difference may result in the state of marmoset ES cells.

iPS Cells

Poster Board Number: F-3201

COORDINATION OF CELL-CELL ADHESION AND MIGRATION BY THE RHO FAMILY GTPASES IS REQUIRED FOR MAINTAINING UNDIFFERENTIATED STATE OF HUMAN IPS CELLS

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Aim: The commitment of stem cells to different lineages is regulated by many cues in the intercellular signals from the microenvironment system. In this study, we found that alterations in E-cadherin-mediated cell-cell adhesion by Rho family GTPase activation can lead to maintain of undifferentiated state of human iPS cells. Methods: The cultures of iPS cells (clone Tic provided from JCRB Cell Bank in Japan) were conducted in medium of ReproStem (ReproCELL, Japan) on the dendrimer surface with d-glucose-displaying with feeder layer of Mitomycin C-treated SNL76/7 cells (ECACC, UK). Here, culture surfaces were designed by immobilizing dendrimer with d-glucose display, that is, 1st-generation (G1), 3rd-generation (G3) and 5th-generation (G5) dendrimer surfaces. To evaluate cell shape and function, immunostaining was performed using the standard protocol. Results: To compare the dynamics of cell behaviors on different culture surfaces, the time-lapse observation

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of hiPS cells on the G1, G3 and G5 surfaces was conducted. After inoculation, the small clumps in all the cultures started to adhere to the surfaces and put out cell extensions exhibiting dynamic protrusions with lamellipodia in colony edge. The cells on the G1 surface showed a round shape with temporal stretching in only colony periphery. In addition, formation of packed colony with clearly defined border was found to occur spontaneously on the G1 surface through cell division as well as migrating cells. With an increase in the dendrimer generation number, however, the cells in iPS colony exhibited active migration, accompanied by cell morphological changes of stretching and contracting. On the G5 surface, however, a frequency of migrating cells in whole colony increased, and maintaining of the cell-cell contacts was appreciably inhibited. From the immunostainings of Rac1 and E-cadherin, the cells in colony edge on the G1 surface expressed Rac1 activation, and appeared E-cadherin expression in the whole colony, indicating that the G1 surface permits the regulation of Rac1 and E-cadherin expression associated with altering in cellular morphology and migratory behaviors. It was also found that OCT3/4expression was, in particular, promoted within iPS colony on the G1 surface, thus supporting the consideration that a balance of Rho family GTPases activation and E-cadherin expression maintains undifferentiated state in hiPS colony. Conclusions: In conclusion, the surfaces of d-glucose-displayed dendrimer regulated the colony formation of iPS cells owing to cell morphological change and migration. The G1 surface is suggested to become an alternative substrate for hiPS colony formation due to the adequate cell migration in hiPS colony. These phenomena can be applied to the maintenance culture of undifferentiated state of hiPS cells based on the adequate balance of Rho family GTPases and E-cadherin activation.

Poster Board Number: F-3202

CHARACTERIZATION OF "DE-UNDIFFERENTIATION" PHENOMENON IN COLONY OF HUMAN INDUCED PURIPOTENT STEM CELLS BASED ON KINETIC ANALYSIS OF SPECIFIC GROWTH RATE

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Introduction: The culture technique for maintenance of human induced pluripotent stem (iPS) cells is not sophisticated owing to extremely high cost and tedious labor, leading to developing issues for the culture environment (ex. feeder-free medium and artificial substrate to remove feeder cells and nature materials, respectively) and the operation (automation system). However, such techniques yield a heterogeneous cell population both between and within colonies, which results in a limited developmental potential and a low production yield. In the present study, we assessed the population balance of undifferentiated and de-undifferentiated states in the culture of iPS colony based on a kinetic growth analysis. Materials and Methods: The cultures of iPS cells (clone Tic provided from JCRB Cell Bank in Japan) were conducted in medium of ReproStem (ReproCELL, Japan) on the gelatin-coated substrate with feeder layer of Mitomycin C-treated SNL76/7 cells (ECACC, UK). The number of cell nuclei in undifferentiated and de-undifferentiated regions was estimated by immunostainings of DAPI (total nuclei) and Oct3/4 (undifferentiated nuclei), and the standard curve of nuclei number per areas of each regions was determined. Results and Discussion: The cultures of human iPS cells on a SNL feeder layer were conducted for culture time of t = 144 h. The majority of iPS colonies grown on the SNL feeder layer showed normal morphology with typical characteristics of hiPS cells with large nuclei with surrounding cytoplasm, the cells being packed tightly together. In some part of a culture vessel, however, some colonies appeared two cell types with different cell morphology in the central and peripheral regions in a single colony. The cells in the center were elongated, usually oriented in spirals or rays towards the center, often with indistinct or ragged cell boundaries. This phenomenon was termed as "de-undifferentiation" associated with the early-stage differentiation of iPS cells. For the quantitative estimation of population balance of undifferentiated and de-undifferentiated states in a single de-undifferentiated colony, the cell number was determined through measurement of each area between undifferentiated and de-undifferentiated regions on the images captured at t = 24 h and 144 h. In the early phase t = 72 h, the de-undifferentiated cells in central of hiPS colony appeared and increased 3 times at the end of culture time t = 144 h. In addition, the specific growth rate in de-undifferentiated region became 5.0×10-2 h-1, indicating specific growth rate of undifferentiated cells is approximately equal to specific growth rate of de-undifferentiated cells. From the results, it is suggested that the differentiation transition process from undifferentiated to de-differentiated states lead to de-undifferentiating phenomenon in hiPS colony during culture. Conclusions: A novel method was established to characterize the kinetics population balance of undifferentiated and de-undifferentiated states in cultured hiPS colony. This quantitative analysis was proposed as a useful tool to assess the phenomenon of de-undifferentiation in hiPS colony.

Poster Board Number: F-3203

MONITORING AND ROBUST INDUCTION OF INTERMEDIATE MESODERM FROM HUMAN IPSCS AND ESCS

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The differentiation method from human pluripotent stem cells, such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), into kidney lineage remains to be developed. Kidney is derived from one of the early embryonic germ layers, intermediate mesoderm (IM), and directing pluripotent stem cells into IM lineage is a crucial step for kidney regeneration. Here we have efficiently generated human iPSC lines that contain an allele of OSR1 gene, a specific marker for IM, into which a green fluorescence protein (GFP) gene was knocked-in by homologous recombination using bacterial artificial chromosome (BAC)-based vectors. We have also established a robust induction protocol using combinational treatment of growth factors, which produces up to 90% OSR1+ cells. These cells expressed other IM marker genes, and could differentiate into multiple cell types included in IM derivative organs, such as kidney, gonad and adrenal cortex in vitro and *in vivo*. Furthermore, the OSR1+ cells can form three-dimensional tubular structures in vitro. These results suggest that our differentiation protocol can induce human pluripotent stem cells into IM cells with similar developmental potential to that in embryos, thereby supplying an unprecedented system to elucidate the mechanisms of IM development and potentially providing a cell source for kidney regeneration.

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Poster Board Number: F-3204

THE CLONAL DIFFERENCE IN RESPONSE TO ATP OF HUMAN INDUCED PLURIPOTENT STEM CELL-DERIVED NEURONS

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Human induced pluripotent stem cells (hiPSCs) hold potent possibility for drug screening and disease modeling. So far, most papers have studied the neuronal differentiation of hiPSCs based on the expression of neuronal markers and their morphology. However, the functional expressions of L-glutamate (Glu) receptors and P2 receptors have not been confirmed sufficiently. We have induced the neuronal differentiation of the neurospheres derived from two clones of hiPSCs (201B7 and 253G1) and confirmed the expression of Tuj1 in both of them at the differentiation day 20. We investigated Ca^{2+} responses of these clones to L-Glu and adenosine triphosphate (ATP) by fura-2 imaging method. 201B7-derived neurons showed Ca^{2+} response to L-Glu at the differentiation day 30, whereas 253G1-derived neurons showed Ca^{2+} response to L-Glu at the differentiation day 10. Of note, 201B7-derived neurons showed $Ca2^{+}$ response to ATP at the differentiation day 20, however, 253G1-derived neurons did not show the response to ATP even at the differentiation day 40. These results suggest that the 201B7 hiPSC clone is suitable for the study about the role of purinergic signaling in the neuronal differentiation of hiPSCs. Currently, we are investigating the time course of P2 receptor-expression of 201B7-derived neurons.

Poster Board Number: F-3205

IPS CELL PRODUCTION FROM HUMAN PERIPHERAL BLOOD STEM CELLS BY OVER EXPRESSION OF NANOG

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Embryonic stem (ES) cells were previously thought to be the best material for transplantation, however many hurdles such as immune-rejection, source and ethical issues have re-directed research towards finding other sources of material. Adult stem cell could be an excellent candidate alternative to ES cells. However, they have a limited capability to produce different cell lineages. Thus, cellular modification to revert adult somatic cells back to an embryonic state is needed. The ability to modify cells through a dedifferentiation process is challenging. However, we have previously shown that over-expression of a single gene, Nanog, responsible for maintaining the embryonic state, in human bone marrow derived mesenchymal stem (MS) cells is enough to induce pluripotency and create ES cell like induced pluripotent stem (iPS) cells. These Nanog over expressing cells showed significantly higher expression of other ES cell genes, including Oct-4, and Sox-2. It was also found that these cells trans-differentiated into neural cells and were immunopositive for β-III tubulin and glial acidic protein, lineage markers for neurons and astrocytes, respectively. *In vivo* studies further demonstrated that the cells were able to survive, migrate and express neural markers after transplantation. Following our study, many groups have successfully produced iPS cells using over expression of multiple embryonic stem cell genes including Nanog, Oct-4, and Sox-2. Peripheral blood stem (PBS) cells have been considered an alternative material to bone marrow cells for bone marrow replacement treatments due to a simplified isolation process, which eliminates the need for bone marrow aspiration from a patient. Thus, PBS cell may be a good starting material alternative to MS cells for generating iPS cells. To further elucidate modified stem cell technology, we proposed to use PBS cells as the starting material to produce modified cell lineages via over-expression of Nanog. Following the induction of pluripotency, we directed differentiation of the cells using an established method of co-culturing with differentiated human neural stem cells. The results of this study showed that PBS cells derived iPS cells capable of exhibiting neural cell-like characteristics based on morphology, cell markers, and gene expression. Through the creation of iPS cells from a non-invasive source, and their subsequent directed neuronal differentiation, we have demonstrated the potential of PBS cells as a starting material for iPS cell technology and future autologous regenerative therapies.

Poster Board Number: F-3206

TAP2-DEFICIENT HUMAN IPS CELL-DERIVED MYELOID CELL LINES AS CELL SOURCE FOR DENDRITIC CELLS FOR IMMUNOTHERAPY

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We established a method by which we can obtain a large number of functional dendritic cells (DC) with a easy procedure from human iPS cells. We transduced iPS cell-derived CD11b+ myeloid cells with genes associated with proliferative or anti-senescence effects. This made the cells capable of propagating for more than 4 months in an M-CSF-dependent manner while retaining their capacity to differentiate into functional DC. We named the iPS cell-derived proliferating myeloid cells "iPS-ML", and the iPS-ML-derived dendritic cells "ML-DC". In addition, we generated TAP2-deficient iPS cell clones by zinc finger nuclease-aided targeted gene disruption. TAP2-deficient iPS cells and iPS-ML avoided recognition by pre-activated allo-reactive CD8+ T cells. The TAP2-deficient ML-DC expressing exogenously introduced HLA-A2 genes stimulated HLA-A2-restricted MART-1-specific CD8+ T cells obtained from HLA-A2-positive allogeneic donors, resulting in generation of MART-1-specific CTL lines. TAP-deficient iPS-ML introduced with various HLA class I genes may serve as an unlimited source of DC for vaccination therapy. If administered into allogeneic patients, ML-DC with appropriate genetic modification may survive long enough to stimulate antigen-specific CTL and, after that, be completely eliminated. Based on the present study, we propose a DC-producing system, which is simple, safe, and applicable to any patients irrespective of their HLA types.

Poster Board Number: F-3207

GENERATION OF CF PATIENT DERIVED IPS CELLS AND EFFICIENT ZFN BASED GENE TARGETING IN TRANSGENIC HUMAN IPS CELLS

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Gene targeting by homologous recombination via customized zinc-finger nucleases (ZFN) is a powerful method to manipulate the genome and correct genetic defects. Clinically applicable ZFN-based gene correction in patient-specific cells was hardly possible so far, due to the inability to sufficiently expand most adult (stem

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and progenitor) cells *in vitro*. However, the availability of human induced pluripotent stem (hiPS) cells with their almost unlimited potential for proliferation and differentiation now offers novel opportunities for the development of patient-specific regenerative therapies. The generation and genetic correction of Cystic Fibrosis (CF) patient specific iPS cells using the ZFN technology and the differentiation of the amended iPS cells towards bronchiolar epithelial cells could be a promising therapeutical approach. For the generation of patient-specific iPS cells endothelial cells from the peripheral blood of CF-patients were isolated and reprogrammed through lentiviral overexpression of pluripotency factors. The CF-iPS cells morphologically resemble human embryonic stem cells, express pluripotency markers and could be differentiated *in vitro* into derivatives of all three germ layers. Aiming at the development of a general ZFN-based gene targeting approach, transgenic human iPS cell clones stably expressing eGFP under an ubiquitous promoter and an appropriate eGFP targeting vector encoding for RedStar were generated. Successful gene targeting resulted in eGFPneg RedStarpos human iPS cells and could be achieved with high efficiencies of up to 1% without any antibiotic selection. This efficient targeting protocol now serve as the basis for further targeting strategies, including functional correction of the Δ508 mutation, and the integration of a CFTR reporter construct into the "safe harbor locus" AAVS1 as important tool for optimization of respiratory differentiation protocols and enrichment of CFTR-expressing epithelial cells. The combination of patient-specific iPS generation and our efficient ZFN targeting protocol could enable the development of innovative cell replacement therapies for various genetic diseases.

Poster Board Number: F-3208

COMPREHENSIVE ANALYSIS ON STABILITY OF HUMAN INDUCED PLURIPOTENT STEM CELLS FROM DECIDUA-DERIVED MESENCHYMAL CELLS

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Induced pluripotent stem cells (iPSCs) may represent an ideal cell source for future regenerative medicine and other aspects of clinical applications. Many types of cells have been successfully reprogrammed into iPS cells and suggested as a promising candidate of cell sources for iPSC generation. We have isolated decidua-derived mesenchymal cells (DMCs), which consists of maternal origin cells, from placenta tissue, and reported their unique properties and advantages for clinical use. We have also concluded that generation of iPSC from DMCs (DMC-iPSCs) provides an opportunity to establish iPSCs-banking systems, which will increase the feasibility of using allogeneic iPSCs with wide HLA variation for clinical applications. Here we demonstrate the stability of DMC-iPSCs under prolonged passages from comparison of genome-wide expression profiles with human embryonic stem cells (hESCs) and sustainable pluripotency both in vitro and *in vivo*, focusing the epigenetic status of X chromosome in DMC-iPSCs both undifferentiated and differentiated state. One of the noncoding genes acting on X-chromosome inactivation, XIST indicates the epigenetic status of female cells. In DMC-iPSC clones, both continuous inactivation and reactivation of X chromosome after reprogramming, were observed respectively. We also observed the loss of XIST expression under differentiated state, suggesting of an aberrant epigenetic state in prolonged DMC-iPSC culture. Regardless of the epigenetic state of X-chromosome, DMC-iPSC clones exhibited similar genome-wide expression profiles to hESCs, and formed differentiated cells contained all three germ layers both in vitro and *in vivo*. Relevance to the epigenetic state and safety in clinical applications of hiPSCs will be discussed.

Poster Board Number: F-3209

GENERATION OF HUMAN IPS CELLS AND INDUCTION OF THEIR NEURAL DIFFERENTIATION UNDER XENO-FREE CULTURE CONDITION

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The discovery of induced pluripotent stem cells (iPSCs) showing embryonic stem cell (ESC)-like characteristics has tremendously advanced the stem cell research. iPSCs are generated by overexpressing some reprogramming factors in terminally differentiated cells under ESC culture condition. Therefore, this iPSC technology has been used to create *in vitro* new potential source of cells for the treatment of many different degenerative diseases and in the future may also allow patient specific stem cell therapies. However, almost reprogramming protocols involve the use of products of animal origin based on ESC culture condition. In this study, we tried to develop a protocol to generate and maintain human iPSCs that would be entirely eliminated xeno-contamination by replacing other reagents of animal origin (serum, trypsin, gelatin) with their recombinant equivalents. Firstly, we succeeded in development of a xeno-free cell culture media that could support the long-term propagation of human ESCs in an undifferentiated state to a similar as conventional media containing animal origin product. Secondly, in order to eliminate, or at least to reduce, the xenogenic contamination from animal feeder cells, we also derived primary cultures of human dermal fibroblasts (Yub1896) under strict xeno-free conditions (XF-Yub), and we determined whether XF-Yub cells could be used as both the cell source for iPSC generation as well as autologous feeder cells to support iPSC growth. Finally, we used lentiviral particles expressing a polycistronic construct encoding Oct4, Sox2, Klf4, and mCherry to reprogram XF-Yub cells under xeno-free conditions. A total of 4 xeno-free human iPSC lines (XF-iPSCs) were generated, which could be continuously passaged in xeno-free conditions and maintained characteristics indistinguishable from hESCs, including colony morphology and growth behavior. Moreover, these all XF-iPSCs showed human ESC-like characteristics including positive staining for alkaline phosphatase, normal karyotype, and expression of human ESC-like markers including Oct4, Nanog, Rex1, Sox2, TRA-1-60, TRA-1-80 and SSEA-4, and formed embryonic bodies and teratomas containing derivatives of the 3 germ layers, and could as well be readily *in vitro* differentiated into neurons under xeno-free culture condition. Also, levels of N-glycolylneuraminic acid (Neu5Gc), which is a non-human sialic acid capable of inducing an immune response in humans, were markedly decreased in hiPSCs cultured under our xeno-free conditions. Taken together, the results presented here demonstrate that (1) human iPSCs are generated from fibroblasts with 3 factors (Oct4, Sox2, Klf4) under completely animal products-free conditions, (2) these intained on autologous feeders, and (3) differentiated into neurons under xeno-free culture condition. Our xeno-free culture system provides a path to a scalable good manufacturing practice

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(GMP)-applicable process of generation of iPSCs for therapeutic applications, and can facilitate the establishment of clinical-grade iPSC lines in suitably "defined" xeno-free culture conditions.

Poster Board Number: F-3210

CENTROSOME AMPLIFICATION IN HUMAN INDUCED PLURIPOTENT STEM CELLS PROMOTED BY INACTIVATION OF CYCLIN-DEPENDENT KINASE 1

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Centrosomes, the cell's major microtubule organizing centers, are composed of a pair of centrioles surrounded by an amorphous protein cloud termed the pericentriolar material. Centrosomes are tightly regulated by the cell cycle, precisely doubling to ensure cells enter mitosis with only two centrosomes to maintain accurate chromosome segregation at mitosis. Centriole duplication begins at the G1-to-S transition, requiring cyclin E-Cdk2 activity. However, human iPSCs and ESCs have significantly reduced G1 phases (2.5-3 hrs). Pluripotent stem cells (PSCs) maintain high cyclin dependent kinase (cdk)-2 activity to drive them through G1 phase rapidly, thereby avoiding exposure to differentiation signals. Protection of Cdk2 activity in PSCs may be accomplished by specific localization to centrosomes. Recent evidence has also suggested a role for Cdk1 in centrosome assembly in somatic cells, where cdk1 inactivation causes G2/M phase arrest with the assembly of multiple centrosomes and cell growth propagated by the unaffected activity of Cdk2. Here, we explore centrosome amplification in H1 hESCs, human foreskin fibroblast 1 (HFF1) iPS cells and the HFF1 somatic parent line, as well as differentiated lineages when the activity of Cdk1 is inhibited with a chemical inhibitor, RO3306 (100nM-10μM) from 24-72 hrs *in vitro*. Analysis of various concentrations of RO3306 on all cell types demonstrated effects on cell cycle arrest and centrosome amplification at concentrations ≥10 μM. H1 ESCs treated for up to 72 hrs with RO3306 did not arrest their cell cycles in G2/M, although nascent differentiation of H1 colonies was detected as early as 24 hrs post drug treatment. Curiously, H1 Cdk1 inhibition did not show appreciable centrosome amplification at either interphase or mitosis after 72 hrs of RO3306 when differentiation of colonies was pronounced. HFF1 parent fibroblasts showed classic cell cycle arrest at G2/M after just 24 hrs of 10μM RO3306 and a nearly 47% increase in interphase centrosome amplification by 72 post drug treatment. Similar results on cell cycle arrest and multiple centrosome formation were observed in cells derived from HFF1 iPS pluripotent colonies differentiated *in vitro*. However, pluripotent HFF1 iPS colonies treated for 24 hrs with 10 μM RO3306 cdk1 inhibitor did not arrest at G2/M despite beginning colony differentiation, and centrosome amplification increased 61% at interphase and 87% in mitotic spindles. Localization of amplified centrosomes showed tight nuclear association in H1 ESC and HFF1 iPS cells, but more robust cytoplasmic centriole formation in HFF1 somatic or HFF1 iPS differentiated cells, with some demonstrating no microtubule assembly at supernumerary centrioles. Collectively, inactivation of Cdk1 in pluripotent iPS cells does not affect cell cycle progression but does increase supernumerary centriole assembly, suggesting a unique interplay between the centrosome and the cell cycle in pluripotent stem cells. Supported by the NIH.

Poster Board Number: F-3211

HUMAN INDUCED PLURIPOTENT STEM CELL ON TEMPERATURE-RESPONSIVE CULTURE DISH CAN BE PASSAGED WITHOUT THE USE OF ENZYME OR MANUAL SELECTION

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Human induced pluripotent stem (iPS) cells have unlimited proliferative potential and the ability to differentiate into any lineage of the three germ layers. Due to the nature of the human iPS cell growth characteristics, these cells always grow as clumps to maintain their stemness and prevent apoptosis. Temperature-responsive culture dish surfaces have been designed to respond to changes in temperature. They allow the release of adherent cells by a simple reduction of the temperature of the cell culture. The covalently immobilized polymer poly (N-isopropylacrylamide) (PIPAAm) forms an even and thin layer on the surface of the culture dish. The PIPAAm layer is slightly hydrophobic at 37°C, allowing the cells to attach and grow on it. When the temperature of the culture is reduced to below 32°C, the PIPAAm layer becomes hydrophilic, binds water and swells, resulting in the release of adherent cells from the culture surface. In this study, we demonstrated that harvesting and passaging human iPS cells are easier by using temperature-responsive culture dishes, rather than by a conventional manual dissection method. Additionally, the iPS cells cultured on temperature-responsive culture dishes maintained their undifferentiated state and pluripotent ability as well as the enzymatic method. After over 2 months of culture on the temperature-responsive culture dishes, the expression levels of pluripotency markers such as Oct-4, Nanog, Tra-1-60 and SSEA4 are maintained. The results of embryoid body forming assay also reveal that the cells can effectively differentiate into different cells of three germ layers in vitro and *in vivo*. In conclusion, the cultivation of iPS cells on temperature-responsive culture dishes was suitable for the maintenance of undifferentiated state and pluripotency.

Poster Board Number: F-3212

GENOME-WIDE GENE EXPRESSION AND DNA METHYLATION ANALYSES OF PARENTAL IMPRINTING IN HUMAN PARTHENOGENETIC IPS CELLS

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Parental imprinting is a phenomenon affecting human development and disease. In order to analyze the role of parental imprinting in human embryogenesis, we generated parthenogenetic human induced pluripotent stem cells (PgHiPSCs), by reprogramming of parthenogenetic ovarian teratomas. PgHiPSCs show typical characteristics of pluripotent cells and have a homozygote diploid karyotype. Global gene expression analysis, using gene expression arrays, of the parthenogenetic cell lines correctly identified most of the known paternally expressed genes. We could demonstrate that a variant of the U5 RNA component of the spliceosome, which is highly enriched in pluripotent stem cells (PSCs), is a novel imprinted gene, as it is down regulated in the parthenogenetic cells, and expressed in a monoallelic fashion in the wild type cells. Our analysis also uncovered multiple miRNAs as novel imprinted clustered

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transcripts, and studied their putative targets. In order to study the consequences of parthenogenesis on human development, we differentiated the PgHiPSCs in vitro and *in vivo*, demonstrating marked effects on the differentiation of the extra-embryonic trophectoderm, and of the embryonic liver and muscle tissues. DNA methylation is considered the hallmark mechanism regulating parental imprinting. To unravel the epigenetic mark of PgHiPSCs, we analyzed the global DNA methylation status of these cells. We have thus performed genome-wide reduced representation bisulphite sequencing (RRBS) on PgHiPSCs and their parental parthenogenetic teratomas, and compared the DNA methylation patterns to a large panel of normal human embryonic stem cells and iPSCs. The analysis correctly identified most of the known imprinted differentially methylated regions (DMRs). Strikingly, our study identified dozens of novel DMRs in both intergenic and intragenic regions. In order to link DNA methylation to gene expression, we have performed RNA high-throughput sequencing in both normal and parthenogenetic HiPSCs. This analysis can potentially classify genes associated with novel DMRs into three classes: A. Genes differentially expressed between PgHiPSCs and normal PSCs, suggesting they are novel imprinted genes; B. Genes that are not differentially expressed between PgHiPSCs and normal PSCs; C. Genes that are not expressed in PSCs. These results suggest that several of the novel DMRs may be associated with cell-type specific imprinted genes or potentially regulate other processes besides gene expression. In the present study we have utilized the iPSC technology to conduct a highly comprehensive analysis of parental imprinting in human embryogenesis. We have thus globally mapped paternally expressed genes, identified multiple novel imprinted non-coding transcripts, correlated the alteration in gene expression with the perturbation in differentiation capacity, and suggested a complex inter-relationship between imprinted DMRs and gene expression. * The first part of this study was recently published in Nature Structural & Molecular Biology

Poster Board Number: F-3213

AUTOMATED DETECTION AND OBSERVATION OF BEATING CARDIOMYOCYTES DERIVED FROM HUMAN IPS CELLS

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Cardiomyocytes differentiated from hES/iPS cells are promising experimental materials for basic research of heart diseases and for drug discovery screening. For functional cardiomyocytes, one indication of differentiation is the beating of a cell or cell clump. However, conventional beat detection methods require the operator to manually scan the culture vessel under a microscope while visually inspecting for beating cardiomyocytes, which is both laborious and inconsistent. In many cases, the cells must also be taken out of the culturing environment for observation / inspection, potentially increasing the risks of contamination and temporal weakening of beating. Such issues cannot be overlooked in the mass production of cardiomyocytes and in applications that require consistent quality and quantitative evaluations. For this reason, we developed an algorithm for automatically detecting beating cardiomyocytes under a culturing environment. Human iPS cells were induced to differentiate into cardiomyocytes while cultured in flat-bottom plates, and observed inside Nikon's BioStation CT. For beat detection, phase-contrast images of the entire culture vessel were captured at 15 fps for a period of 4 seconds. We developed the algorithm to detect the beating of the differentiated cardiomyocytes based on the optical flow method.The developed algorithm detected more than 80 % of beating colonies. On the other hand, the degree of detection by skilled operators greatly varied among individuals, ranging from 55 ~ 91%, indicating the difficulty of quality control by manual detection. The threshold of the algorithm for beat detection can be adjusted, which allows the detection criteria to be set according to the purpose.The algorithm significantly reduced the effort and time to find beating cardiomyocytes used by the operators while eliminating inconsistency due to human operations, thereby contributing to quality management. It is expected that non-invasive automated detection using the algorithm will become an essential tool in drug discovery and regenerative medicine involving hES/iPS cells.

Poster Board Number: F-3214

ANALYSIS OF MICRORNA PROFILES IN HUMAN EMBRYONIC STEM CELLS AND INDUCED PLURIPOTENT STEM CELLS

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MicroRNAs (miRNAs) are noncoding RNAs of approximately 22 nucleotides in length that negatively regulate the post-transcriptional expression by translational repression and/or destabilization of protein-coding messenger RNAs (mRNAs). Recent advances in small RNA research have implicated the importance of miRNAs for self-renewal and differentiation in human embryonic stem (hES) cells and human induced pluripotent stem (hiPS) cells. For example, the knockout mouse ES cell lines that disrupted Dicer-1 or DGCR8, which are essential for biogenesis of miRNA, displayed the decrease in proliferation of ES cells or severe defects in differentiation both in vitro and *in vivo*. However, the genes that were regulated by the ES and iPS specific miRNAs are largely unknown. Here we report the differences in miRNA and mRNA expression profiles among three undifferentiated hES cell lines, eight undifferentiated hiPS cell lines derived from human amniotic cells, fetal lung fibroblasts and placental artery endothelium and their parental cells to understand the roles of miRNAs in maintenance of hES or hiPS cell properties. First, we examined the expression profiles of over 700 miRNAs using both microarray and qPCR analysis to identify the signatures of miRNAs that were up-regulated in hES and hiPS cells. These large scale miRNA expression profiles of hES cells, hiPS cells, and their parental cells indicated that hiPS cells were clustered closely with hES cells but separately from their original cells. We found five miRNAs (miR-302a, miR-302b, miR-302c, miR-302d, and miR-367) that were highly expressed in hES cells and were remarkably up-regulated in iPS cells compared to their parental cells. These miRNAs are conserved cluster and located within an about 700 bp region on chromosome 4 in the antisense intron of the protein-coding gene. Next, we examined the global gene expression profiles of these cell lines using microarray. Comparative profiles of miRNAs and mRNAs from the same samples may reveal the role of miRNAs in iPS cells. We found that several cell cycle regulators, such as CCND1, CDKN1A, CDKN2A and CDKN2C, were remarkably down regulated in iPS cells compared to their parental cells, suggesting that miRNA have an important role in proper cell proliferation in hiPS cells. Here, the important roles of miRNAs in regulating the genes for self-renewal and differentiation will be discussed.

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Poster Board Number: F-3215

GENETICALLY MATCHED HUMAN IPS CELLS REVEALED THAT THE PROPENSITY FOR IPS CELLS TO DIFFERENTIATE INTO CARTILAGE LINEAGE CELLS DIFFERS WITH CLONES, BUT NOT CELL TYPE OF ORIGIN

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Background: For regenerative therapy using induced pluripotent stem cell (iPSC) technology, cell type of origin to be reprogrammed should be properly chosen based on accessibility and reprogramming efficiency. Some reports pointed out that iPSCs exhibited a preference for differentiation into their original cell lineages, though this was argued against in other reports. Therefore, still there has been no conclusion about which type of cell is most appropriate as a source for iPSCs. *Methodology/Principal Findings:* Genetically matched human iPSCs from different origins were generated using bone marrow stromal cells (BMSCs) and dermal fibroblasts (DFs) of the same donor, and the global gene expression profile and differentiation into the chondrogenic lineage of each clone were analyzed. Genes expressed differentially in BMSCs and DFs were equally silenced in our bona fide iPSCs. After cell-autonomous and induced differentiation, each iPSC clone exhibited various differentiation properties, which did not correlate with cell-of-origin. *Conclusions/Significance:* The reprogramming process may remove the difference between DFs and BMSCs at least for chondrogenic differentiation property. Qualified and genetically matched human iPSC clone sets established in this study are valuable resources for further basic study of clonal differences.

Poster Board Number: F-3216

DISTINCT MECHANISMS UNDERLYING MULTIPLE ENHANCERS OF HUMAN CELLULAR REPROGRAMMING

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A minimal set of reprogramming factors consisting of OCT3/4, SOX2, KLF4 and c-MYC is sufficient to generate induced Pluripotent Stem (iPS) cells from somatic cells, but at very low efficiency. Efforts to increase efficiency have identified several factors that enhance the process of reprogramming. However, the modes of action for each factor in reprogramming and relationship between them remains unclear. Here, we directly compared the reprogramming enhancers NANOG, LIN28, Cyclin D1 and short hairpin RNA-mediated suppression of p53 (p53 shRNA) during human cellular reprogramming.Cyclin D1 and p53 shRNA increased the proliferation of both non-reprogrammed and reprogrammed cells in human fibroblast cultures. On the other hand, LIN28 specifically enhanced the proliferation of only reprogrammed cells. Noteworthy, LIN28 increased the proportion of reprogrammed cells even in absence of cell proliferation, which was not the case for cell cycle regulators. Indeed, in proliferating fibroblasts, LIN28 accelerated the progression of reprogramming and promoted proliferation of reprogrammed cells with an advanced time course compared with cell cycle regulators. Interestingly, the early reprogramming activity of LIN28 was accompanied by enhanced downregulation of fibroblast-expressed genes. In contrast to the other factors, NANOG exhibited no positive effects on reprogramming of human cells as an enhancer in combination with with OSKM. Therefore, we conclude that these reprogramming enhancers act on apparently distinct targets during human cellular reprogramming.

Poster Board Number: F-3217

GENERATION OF EXOGENOUS GENE-FREE HUMAN INDUCED PLURIPOTENT STEM CELLS FROM PERIPHERAL BLOOD MONOCYTES USING A DEFECTIVE AND PERSISTENT SENDAI VIRUS VECTOR INSTALLED SIMULTANEOUSLY WITH OCT4, SOX2, KLF4, AND C-MYC

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Purpose: Human induced pluripotent stem (iPS) cells have been generated from a variety of tissue cells via reprogramming by ectopic expression of defined factors. Potentially, a monocyte prepared from peripheral blood is an ideal source for generating human iPS cells for the following reasons. First, the monocytes can be isolated aseptically from human peripheral blood without invasive treatment. Second, unlike T and B lymphocytes, the monocytes retain the complete human genome without rearrangement of the T cell receptor subunits loci or the IgH locus, ensuring the generation of safer human iPS cells. However, monocytes have not been considered a practical source for reprogramming because they cannot proliferate under *in vitro* culture conditions and active cell proliferation is considered critical for reprogramming. Extremely low transduction capacity also hampers the use of the monocytes for reprogramming. We recently developed a novel RNA virus vector (replication-defective and persistent Sendai virus vector; SeVdp) capable of stable gene expression for more than six months without chromosomal integration. We found that SeVdp-iPS harboring Oct4, Sox2, Klf4 and c-Myc cDNAs together on a single vector can generate exogenous gene-free iPS cells efficiently (reprogramming efficiency ~1%) from primary fibroblasts (Nishimura, et al., J. Biol. Chem., 286, 4760). In the present study, we report that SeVdp-iPS could reprogram human peripheral blood monocytes to become iPS cells. Results: CD14-positive monocytes were isolated from human peripheral blood mononuclear cells with anti-human CD14-magnetic beads. The monocytes were infected with SeVdp-iPS at room temperature for 2 hours (MOI = 3) and were seeded onto feeder cells. On the fifth day after infection, discernible colonies emerged (reprogramming efficiency ~0.5%). Subsequent treatment of the cells with siRNA against SeV L gene eliminated the SeVdp-iPS genome by interfering with transcription/replication of the vector. Finally, human monocyte-derived exogenous gene-free iPS (M-iPS) cells were established within 30 days after infection. Gene rearrangement analysis of nine M-iPS cell clones revealed that their genome showed no rearrangement of the T cell receptor subunits loci or the IgH locus, indicating that the M-iPS cells were not derived from lymphocytes. M-iPS cells differentiated into a teratoma with all three germ layers, whereas these cells preferentially redifferentiated into blood cell lineages on stromal cells *in vitro*. Our

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findings suggest that reprogramming monocytes with SeVdp-iPS is an attractive strategy for generating exogenous gene-free human iPS cells with a complete genome. These properties may be useful for fundamental research and in future clinical work.

Poster Board Number: F-3218

ESTABLISHMENT OF A HIPSC LIBRARY OF THE BRAZILIAN POPULATION: A TOOL FOR *IN VITRO* CLINICAL TRIALS

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In addition to their potential use in regenerative medicine, pluripotent stem cells are an invaluable experimental *in vitro* system for disease modeling, for the screening of new drugs, and for testing drugs´ efficacy and toxicity. Currently, new drugs are licensed on the basis of efficacy in only 30% of the subjects tested. Moreover, these drugs are tested in general populations in Europe and North America, and are sold in developing countries without the knowledge of how effective or safe they are for these populations. On the other hand, the need to test drug response in different populations represents an economic and strategic challenge to the pharmaceutical industry, and therefore the need to develop *in vitro* systems where to perform such studies. The establishment of a cellular library that represents the genetic diversity of a specific population could be an alternative way of testing drugs for this population before clinical trials. In particular, induced pluripotent stem cells (iPSCs) are a powerful type of cells for these studies, since one can derive multiple differentiated cell types from them. The Brazilian population is one of the most heterogeneous worldwide, a result of its history of colonization and five centuries of interethnic crosses among Europeans (mostly Portuguese), Africans and Amerindians. Therefore, our objective is to develop a library of hiPSC that represents the genetic heterogeneity of the Brazilian population to be used for *in vitro* studies of drug response and toxicity. Peripheral blood will be collected from 2,000 randomly chosen individuals from the collection of 15,105 civil servants enrolled in The Brazilian Longitudinal Study of Adult Health (ELSA - Brasil, www.elsa.org.br), a multicenter cohort project to investigate incidence and risk factors to chronic pathologies like diabetes and cardiovascular diseases in the Brazilian population. For each participant, a database of exams was established, including clinical and anthropometric examinations, a 12-lead resting electrocardiogram, measurement of carotid intima-media thickness, analysis of heart rate variability, echocardiography, measurement of pulse wave velocity, hepatic ultrasonography, retinal fundus photography, an oral glucose tolerance test, and overnight urine collection. Reprograming of erythroblasts will be performed according to Chou et al. (Cell Res., 21(3):518-29, 2011). Genetic ancestry of each participant will be determined by SNP analysis, and polymorphisms related to drug metabolism will be genotyped with the DMET ARRAY SYSTEM (Affymetrix). Initially, 400 individuals will be selected for reprogramming according to their genotypes. Three independent hiPSC lines for each participant will be established. Within four years we expect to have a set of genetically diverse hiPSCs from the Brazilian population to be used for *in vitro* clinical trials of new drugs in this population. In addition, since each cell line will be connected to a database of clinical parameters, this library may also enable the identification of cellular phenotypes related to particular clinical manifestations. Funding by BNDES, FINEP, MCT/CNPq and MS/DECIT.

Poster Board Number: F-3219

EVALUATION OF HUMAN IPS CELLS BY NEURAL DIFFERENTIATION AND TUMORIGENICITY

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Neural stem/progenitor cells (NS/PCs), derived from human embryonic stem (hES) cells and human induced pluripotent stem (hiPS) cells, have been expected to be applied for regenerative medicine, and for in vitro modeling for the research on neural development and pathophysiological analysis of neurological disorders. However, each hiPS cell line, even those derived from identical somatic cells, has distinct pluripotent property and differentiation propensity. Therefore, we have to evaluate each hiPS cell line to obtain safe NS/PCs with normal physiological properties to avoid tumorigenesis and inappropriate pathophysiological analysis due to abnormal differentiation. In this study, we prepared NS/PCs from hES cells and hiPS cells as neurospheres, and examined their differentiation potentials, functional properties, and gene expression profiles in vitro. We also examined the proportion of residual undifferentiated cells in derived NS/PCs, and the expression of retroviral transgenes, which had been used for the establishment of hiPS cells, during the neural differentiation, to estimate their tumorigenic potentials. Finally, we injected hES, hiPS cell-derived NS/PCs into brains and testes of NOD/SCID mice to evaluate their differentiation properties and tumorigenicities *in vivo*. Through these analyses, we found that one of the hiPS cell lines we used could not differentiate to form neurospheres efficiently, and that NS/PCs derived from some of the hiPS cell lines formed tumors after transplantation, but without teratoma formation. Focusing on these distinct properties of hiPS cell lines, we further analyzed the underlying differences among hiPS cell lines, and evaluated the quality of hiPS cell lines. In the present study, we could obtain relatively safe and normal hiPS cells for the application for varieties of researches. However, we had to perform above mentioned multiple, complex, and time-consuming experiments. In the near future, we need to develop much easier and faster screening system to evaluate the qualities of hiPS cells.

Poster Board Number: F-3220

DIFFERENTIAL MESODERM PROPENSITIES OF FEEDER-FREE DERIVED HUMAN IPSC.

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Somatic cells reprogramming was achieved by transducing human foreskin fibroblasts with the previously described canonical human pluripotent transcription factor Oct4, Sox2, Nanog and Lin281. Thirteen iPS cells clones were derived and two clones were selected for further characterisation, hiPS-drg9 and hiPS-drg13. The two clones hiPS-drg9 and hiPS-drg13 exhibited typical human ES like colony morphology at early passage (p1) and improved with successive passages. The human iPS clones were maintained under feeder free

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matrigel conditions and were sustained with MEF-CM supplemented with bFGF and IGF-II. Further characterisation show that these lines show good self-renewability in culture (p30 currently), and express all the surrogate embryonic stemness markers. Like the human ES cells line ES-4, hiPS-drg9 and hiPS-drg13 presented pluripotent markers like Oct4, SSEA3 and 4, Tra1-60, Tra1-81 and IGF1R. In general both clones show good self-renewability, presented normal genome (karyotype and CGH analysis) and formed teratoma with the three germ layers. Importantly, the two clones exhibit similar IGF1 receptor undifferentiated colony-initiation potential as described previously with many human ES cell lines2, showing the importance of the IGF1R/IGF-II signalling axis even in the induced pluripotent stem cells system, which has never been described before. Interestingly the two lines exhibit differences in the potential to *in vitro* develop into the cardiac and blood cell fates. The hiPS-drg9 clone has better propensity to blood development while hiPS-drg13 gives rise better to the cardiac cell fate. Hematopoietic differentiation potential was determined by the ability of EBs exposed to blood inducing factors to give rise to the bipotential hemogenic endothelial precursor (CD31posCD45neg), the hematopoietic progenitors (CD34posCD45pos) and the committed blood cells population (CD45pos). Cardiac development was determined by the ability of the hiPS-drg9 and hiPS-drg13 EBs to give rise to the cardiogenic KDRlow/c-Kitneg fraction (I) levels (data not shown) and for selected cardiac differentiation markers, Gata4, Troponin I and Nkx2.5 by flow cytometry.

Poster Board Number: F-3221

DIFFERENTIATION OF HUMAN INDUCED PLURIPOTENT STEM CELLS BY CELL PATTERNING.

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Cultivation of human induced pluripotent stem cells is carried out on feeder cells or cell adhesive proteins. When guiding differentiation, embryoid bodies are made in culture solution containing differentiation-inducing factors and maintained for a fixed period with adhesive cultivation. It has been reported by researchers in the regeneration medicine field of an eye in recent years that stem cells not only differentiate into the cell of the purpose part, but become the structure of an organization itself. In that case, the three-dimensional cell culture like the target structure is performed. In other ways, there is also a report that the directivity in early stages of differentiation is controlled by the cell adhesion domain in the two-dimensional plane. But, those methods have problems cells are added various differentiation-inducing factors at different times and take three or four months to differentiate into other cells. In order to solve such problems , development of cell culture materials has attracted attention and the effect which various cultivation environments gave to human induced pluripotent stem cells has been reported. In our research, the effect of patterned cell culture was proved by the following methods. At first, cover glass was coated with poly ethylene glycol, and its surface became non adhesive. Next, cell adhesive region and non adhesive region were produced by lithographic exposure. In short, UV radiation decomposed poly ethylene glycol and substrate became cell adhesive. Proteins absorbed on cell adhesive region, not on non adhesive region. by XPS and immunostaining. Human induced pluripotent stem cells and human embryonic stem cells adhered and maintained stemness. Moreover, after human induced pluripotent stem cells had been incubated on various diameter circle patterned substrate for 3weeks, RNA was extracted from each patterned cells, and quantitative PCR revealed that some genes expression changes.

Poster Board Number: F-3222

PROTEOMIC ANALYSIS-BASED IDENTIFICATION OF PROTEINS RELATED TO DIFFERENTIATION OF HUMAN IPS CELLS INTO CARTILAGE CELLS

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Induced pluripotent stem cells (iPSCs) are expected to contribute greatly to regenerative medicine and disease treatment. Each of the established iPSC exhibits different properties, such as differentiation and proliferation capacities. It is unclear how the properties of iPSCs are regulated, and there are no markers for discriminating the properties of each iPSC. Therefore, it is important to understand the regulatory mechanism and identify biomarkers for the properties of iPSCs. In this study, we examined the protein expression patterns of human iPSC clones that exhibited various differentiation properties for chodrocyte-lineage. Proteins extracted from cultured human iPSCs (approximately 1×10^6) were subjected to proteome analysis using two-dimensional electrophoresis. Protein spots with altered expression levels (>2-fold) were subjected to peptide mass fingerprinting or MS/MS analyses with tandem mass spectrometry for protein identification. The proteins with altered expression levels were classified into categories based on their subcellular localization and function. Several proteins identified in this study were localized to the extracellular or plasma membrane. In particular, extracellular proteins have the best chance of being secreted into the medium culture, suggesting that these proteins may be excellent candidates for non-invasive markers indicating differentiation property, although further study is required.

Poster Board Number: F-3223

EFFICIENT DERIVATION, MAINTENANCE AND CHARACTERIZATION OF HUMAN INDUCED PLURIPOTENT STEM CELLS (HIPSCS) AT THE CEDARS-SINAI IPSC CORE FACILITY

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Introduction: The Cedars-Sinai Regenerative Medicine Institute iPSC Core Facility provides pluripotent stem cells (PSCs) for research purposes. Each patient iPSC line derived from skin fibroblasts is expanded, banked and rigorously characterized by the Core prior to distribution under both feeder and feeder-free conditions for a fee. The core also specializes in generation of patient iPSC lines from other somatic cells, such as corneal limbal, gut, blood, and adipose cells. The distributed iPSC lines are accompanied with a Certificate of Analysis (COA) confirming pluripotency, lack of integration and normal karyotype, as well as assessment of differentiation potential to three germ layers using embryoid body and teratoma formation and gene expression assays. Methods: Patient iPS cell lines were generated using Amaxa (Lonza) and Neon (Invitrogen) transfection systems. Reprogramming was performed under 5% O2 by transfecting parent cells with non-integrating oriP/EBNA1 plasmids that rely on episomal expression of six factors: OCT3/4, SOX2, KLF4, L-MYC, LIN28, and p53 shRNA. Cultures were then plated on Matrigel and fed mouse embryonic fibroblast conditioned media supplemented with small molecules, PD0325901, A-83-01, CHIR99021, and Sodium Butyrate until day 20, after which they

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were switched to mTeSR1 media. Colonies resembling PSC morphology were picked and plated on Matrigel. Clonal expansion of five to fifteen colonies was performed. Three clones were selected for G-Band karyotyping and further characterization. This protocol has been adapted for generation of iPS cells in a 96-well plate format. Cryopreservation media were compared to assess optimal freeze-thaw of feeder-free iPSCs. Briefly, iPSCs were incubated with ROCK inhibitor for 1-hour and subsequently cryopreserved in either CryoStor-CS10 (Biolife Solutions) or a cocktail consisting of 30% FBS and 10% DMSO in mTeSR1. Cells were frozen at -80ºC for 24-hours in slow cooling (1ºC/min) containers and transferred to liquid nitrogen in cryovials, which were later thawed and re-plated on Matrigel in mTeSR1. Manual and enzymatic passaging techniques were compared between StemPro EZ-Passaging tool, Dispase and Versene (all Life Technologies). Results: The Core has derived 18 non-integrating iPSC lines from control subjects as well as patients with Spinal Muscular Atrophy, Huntington's disease, Amyotrophic Lateral Sclerosis, MCT8 deficiency, and Neurofibromatosis 1. Amaxa system was able to generate iPSC lines with 2 to 3-fold higher efficiency than Neon system. Chromosomal aberrations were identified in 3/18 (16%) iPSC lines. After cryopreservation, post-thaw iPSC recovery rates showed that CS10 had >50% recovery, while cells frozen in mTeSR1/FBS/DMSO were not viable. Post-passage, the EZ Passaging tool and Versene techniques resulted in cultures with <5% differentiation while Dispase resulted in significant cell differentiation. Conclusion: The Cedars-Sinai iPSC Core Facility facilitates PSC research by providing comprehensive support to the global scientific community for creating relevant human disease models by generating and characterizing non-integrating iPSC lines. We have improved and optimized iPSC generation, passaging and cryopreservation protocols. We have derived excellent quality non-integrating iPSC lines while maintaining a low rate of chromosomal abnormality. Work is now underway to transform our protocols into high-throughput iPSC generation platform.

Poster Board Number: F-3224

EFFICIENT INDUCTION OF HUMAN T LYMPHOCYTE CELL DERIVED IPSCS USING TEMPERATURE SENSITIVE SENDAI VIRUS VECTORS

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Induced pluripotent stem cells (iPSCs) can be generated from somatic cells by exogenous expression of defined transcription factors. Genome integration-free reprogramming using RNA viruses, protein or plasmid vectors for iPSCs induction has recently been reported (Ban et al., 2011; Cho, 2010; Fusaki et al., 2009; Jincho, 2010; Kim, 2009; Okita, 2008; Stadtfeld et al., 2008; Yu, 2009; Zhou, 2009). In particular, Sendai virus (SeV) vectors have been shown to efficiently reprogram fibroblasts and T lymphocytes (Fusaki et al., 2009; Nishimura et al., 2011; Seki et al., 2010) and thus have been suggested as strong candidates for future standard reprogramming vectors for circulating blood cells. However, although SeV is not supposed to become integrated into the host genome, viral particles could persist in iPSCs after induction and modify their functions. Such persistent expression of the four exogenous factors (human c-Myc, Oct3/4, Sox2, Klf4; MOSK) may affect differentiation capacity, tumorigenicity and homogeneity of iPSCs. Because of this problem, it is likely that SeV-mediated iPSCs are not fully pluripotent. In this study, we used an improved version of a temperature sensitive (TS) SeV vector to generate iPSCs in which the SeV could be efficiently depleted by a simple temperature shift. Pluripotency of the resulting iPSCs was validated by generating germ line chimeric mice. And we then showed human T cells were reprogrammed to iPSCs (T-iPSCs), which is also virus-free in the host genome, using TS SeV vector. Furthermore, we showed that efficient induction of human T-iPSC using SV40 combined with defined factors (SV40+MOSK) compared to using defined factors (MOSK). Finally, we induced T-iPSCs from several types of human T cells such as CD4+ or CD8+. These results suggested that the TS SeV was qualified as a future standard vehicle to reprogram human cells for clinical purposes.

Poster Board Number: F-3225

HUMAN INDUCED PLURIPOTENT STEM CELL LINES SHOWED SIGNIFICANT DIFFERENCES IN DIFFERENTIATION PROPENSITY FOR LINEAGE DERIVATIVES

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The generation of induced pluripotent stem cells (iPSCs) from somatic cells provides an invaluable resource for drug or toxicology screening, medical research, and patient-specific cell therapy. In this study, we compared the expression of 25 lineage specific markers in nine human induced Pluripotent Stem cell (hiPSC). Based on the comparative levels of the markers of each lineage, we aimed to hypothesize which of the hiPSC line have a higher intrinsic propensity to differentiate toward specific cell type. The hiPSC lines were derived from three individuals by retroviral transduction of Yamanaka factors. The hiPSCs were induced for differentiation spontaneously by embryoid body formation. Lineage-specific expression of lineage specific markers was analyzed by real-time PCR at three time points (0, 7 and 14 days after differentiation initiation). There was a significant propensity to differentiate into specific lineages among different hiPSC lines even with same genetic background. For example hiPS T1 (drived from a patient with Tyrosinemia disorder) showed the lowest expression of both Mesoderm and Ectoderm lineages compared to hiPS T3 (derived from the same patient) and hiPS4 (drived from a healthy person) cell lines that had highest expression of Ectoderm and Mesoderm lineage markers, respectively. These results may indicate the importance of lineage-specific differentiation screening of hiPSCs before using them in biomedical applications. For exploring correlation of propensity between spontaneous and direct differentiation, hiPSC lines with different differentiation propensity were differentiated into the neural and cardiac induction. The integration of spontaneous and direct differentiation results will be discussed.

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Poster Board Number: F-3226

AUTOLOGOUS CULTURE OF NEURONAL CELL BY THE FEEDER CELLS LAYER AND THE ASTROCYTE-RELEASE FACTORS DERIVED FROM HUMAN INDUCED PLURIPOTENT STEM CELLS

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Human induced pluripotent stem (hiPS) cells have pluripotency and self-renewal capacity. HiPS cells having this property are useful for regenerative medicine and expected to applied to the evaluation system for medical drugs and chemical substances. HiPS cell culture is established several methods for differentiation of neuronal cells. In those methods, proliferation of hiPS cells is shown as formation of the colony on a feeder cell layer of mouse embryonic fibroblast (MEF). For those reasons, a quality of fibroblast layer is believed to affect the proliferation of iPS cells undifferentiated. In order to use in the experiment, MEF is separated from fetal rat. This anatomical operation, it has some ethical problems and the complexity of the technology. For these reasons, as a method of supply of fibroblast cells, the superiority of the MEF can be differentiated from iPS cells will be high. We are to establish a co-culture system of iPS cells it has created a MEF cells derived from the cell iPS. Frequency of colony formation of undifferentiated iPS cells is a guarantee that the good quality of the cells layer derived from hiFB. This undifferentiated degree was determined by a real-time PCR and a immunocytochemistry. The antibody- positive cells for the undifferentiation are shown with antibodies of Oct3/4, Alkaline phosphatase and Nanog, respectively. iPS cells to differentiate into nerve cells in a delicate culture conditions.This indicates the potential to differentiate into cells of three germ layers either by culture conditions. In addition, iPS cell survival is maintained by forming a colony. On the other hand, undifferentiated colonies by a floating culture to form a cell mass which differentiate into neural stem cells of the ectoderm. The culture medium of a floating culture is containing with FGF-2 and astrocytes conditioned medium (Nakayama and Inoue, 2006). The differentiated cells derived from iPS are used as a cell source for creating the culture medium.In this report, the fibroblasts derived from hiPS cells were used as feeder cell layer. Using the autologous cell culture system, we performed a research on nerve cell lineage further.

Poster Board Number: F-3227

HUMAN INDUCED PLURIPOTENT STEM CELL-ENGINEERED TISSUE SHEETS WITH DEFINED CARDIOVASCULAR CELL POPULATIONS AMELIORATE FUNCTION AFTER MYOCARDIAL INFARCTION

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BACKGROUNDS: To realize cardiac regeneration with human induced pluripotent stem cells (hiPSCs), efficient differentiation from hiPSCs to defined cardiac cell populations (cardiomyocytes [CMs]/ endothelial cells [ECs]/ vascular mural cells [MCs]), and transplantation technique for fair engraftment are required. METHODS & RESULTS: Previously, we have reported an efficient cardiomyocyte differentiation protocol based on high density monolayer culture (PLoS One, 2011), in which cardiac troponin-T (cTnT)-positive cardiomyocytes robustly appeared with 50-80% efficiency. In this study, we further modified the protocol to induce vascular cells (ECs/MCs) together with CMs. Vascular endothelial cell growth factor (VEGF) supplementation for induction of ECs resulted in proportional differentiation of cTnT+-CMs (62.7±11.7% of total cells), VE-cadherin+-ECs (7.8±4.9%) and PDGFRb+-MCs (18.2±11.0%) (FACS analysis) at differentiation day 15 (n=12). Then, these cells that induced were harvested and transferred onto temperature-responsive culture dishes (UpCell dishes; CellSeed, Tokyo, Japan) to fabricate cardiac tissue sheets including defined cardiac populations (CMs/ECs/MCs). After 4 days of culture, we successfully collected self-pulsating cardiac tissue sheets consisted of CMs (46.9±15.9% of total cells), ECs (4.1±3.7%), and MCs (22.5±15.7%) (FACS analysis) (n=12). Multichannel extracellular potential analysis revealed that the cardiac tissue sheets hold unidirectional and regular electrical propagation, with no ectopic foci (MED 64 system). A regular calcium transient was observed throughout the sheet along with spontaneous beating. Tri-layered hiPSC-derived cardiac sheets were transplanted to a myocardial infarction (MI) model of athymic rat heart one week after coronary ligation. In transplantation group, echocardiogram (4 weeks after transplantation) showed a significant improvement of systolic function of left ventricle (fractional shortening: 20.9±5.5vs39.4±6.5%, $p<0.001$, n=11) and a decrease in akinetic area (18.1±10.8vs1.4±3.1%, $p<0.001$, n=11) (pre-treatment vs 4weeks after transplantation). Such effects were sustained for more than 3 months. CONCLUTIONS: Transplantation of hiPSC-derived cardiac tissue sheets significantly ameliorates cardiac function after MI. Thus, we developed a valuable technological basis for hiPSC-based cardiac cell therapy.

Poster Board Number: F-3228

EFFICIENT HUMAN IPS CELLS INDUCTION USING MAPK INHIBITORS

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Induced pluripotent stem (iPS) cells can be generated from mouse and human fibroblasts by the retrovirus-mediated transfection of four transcription factors, namely Oct3/4, Sox2,c-Myc, and Klf4. iPS cells have large potential on regenerative medicine. However, the induction efficiency of iPS cells is very low and the underlying mechanisms are largely unknown. More efficient methods for iPS cells generation will be desirable. Mitogen-activated protein (MAP) kinases are serine/ threonine-specific protein kinases that respond to extracellular stimuli (mitogens, osmotic stress, heat shock and proinflammatory cytokines) and regulate various cellular activities, such as gene expression, mitosis, differentiation, proliferation, and cell survival/apoptosis. Here I screen MAP kinases inhibitors and generate human iPS cells using inhibitors. One inhibitor has strong effect to promote human iPS cells induction. I try to clarify this phenomenon using microarray analysis and suppressing downstream gene. Some genes are picked out as a strong candidate gene. Our results provide insights for mechanisms of reprogramming and tools of efficient human iPS cell generation.

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Poster Board Number: F-3229

GENERATION OF GMP GRADE HUMAN INDUCED PLURIPOTENT STEM CELLS

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Induced pluripotent stem cell (iPSC) represents a major source of patient-specific cells with the potential to differentiate into all the cell types of the human body. Given its significant usefulness in basic and clinical research, as well as in clinical applications, the demand for human iPSC (hiPSC) keeps on increasing. However, the process of developing and characterizing iPSC is complicated, and the overall procedures/protocols/reagents for hiPSC generation vary significantly among different laboratories, leading to big concerns about the product safety, quality, effectiveness, and consistency. Moreover, in order to generate clinical-grade hiPSC with defined quality and safety characteristics that are safe for patient, good manufacturing practice (GMP) needs to be applied from starting material preparation to product test. Based on GMP regulations, all cells must be isolated and cultured under defined conditions without xenogeneic reagents. As such, the use of standard bovine serum and the other source of tissues, which are commonly involved in human iPSC generation, should be prohibited. In addition, the transcription factors used in reprogramming would also have to be prepared under GMP conditions. Therefore, modifications of current protocols to generate hiPSC are urgently needed. To this end, we optimized the conditions, and create several patient-specific iPSC lines consistently in a defined condition without xenobiotic factors (feeder- and serum-free). In order to determine the hiPSC quality, the molecular and physiological properties of these hiPSC lines were further compared to that of hiPSC lines generated under standard condition. This study provides inside into the potential of streamlining the quality control process that will ultimately accelerate their application in process of stem cell therapy.

Poster Board Number: F-3230

AP-1 TRANSCRIPTION FACTOR JDP2 INDUCES THE REPROGRAMMING OF HUMAN MEDULLOBLASTOMA TO GENERATE INDUCED PLURIPOTENT STEM CELLS

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Transcription factor Jun dimerization protein (JDP2) plays roles in cell cycle regulation, cellular senescence, nuclear reprogramming and oncogenesis through the epigenetic control involved in cascades of $p19^{Arf}$-Mdm2-p53-p21-cyclin/CDK or $p16^{Ink4a}$-cyclin/CDK-RB-E2F (1-5). Clinical studies of medulloblastomas indicate that JDP2 might be a tumor suppressor gene candidate because normal granule cells express significant levels of JDP2, whereas cancer cells do not. Thus, we generated three different induced pluripotent stem cells (iPSCs) from human medduloblastoma cancer cells (DAOY1) using Lenti-virus encoded standard 4 factors (4F; Oct4, Klf4, Sox2 and c-Myc), 2 factors (2F; Oct4 and JDP2) and 1 factor (JDP2). The original DAOY1 expressed three standard stemness genes like Oct4, Sox2 and Nanog, but did not show the alkaline phosphatase activity. By contrast, iPSCs expressed stemness genes and demonstrated the alkaline phosphatase activity. Moreover, we found that iPSCs reduced the tumor progression as compared with DAOY1 in SCID mice. These results indicate that JDP2 is highly possible to function as tumor repressor. We also found the role of JDP2 is concerned the signaling of Wnt signals such as the genes encoding LEF1 (lymphoid enhancer binding protein), Wisp2 (Wnt 1 inducible signaling pathway protein 2) and Sfrp2 (secreted frizzled-related sequence protein 2). The cross talk of Wnt signal and LIF/JAK-STAT3-Oct4 will be critical in generating iPS cells and the role of JDP2 in nuclear reprogramming and function as the tumor suppressor in progression into the medulloblastoma

Poster Board Number: F-3231

IDENTIFICATION OF NOVEL CELL SURFACE MARKERS TO ILLUMINATE THE ROUTE TO MOUSE IPS CELLS

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The generation of induced Pluripotent Stem Cells (iPSCs) from somatic tissues has been expected to revolutionise regenerative medicine. However, reprogramming is highly inefficient, thus hampering attempts to accurately investigate the molecular mechanisms of successful reprogramming. Time-course microarray data generated using bulk populations illustrates serial changes of gene expression during reprogramming, but as this data also includes the proportional expansion of early-arising fully reprogrammed cells, gene expression changes occurring during pluripotency induction may be masked. Changes in chromatin modifications upon induction of reprogramming factors have also been characterized, however only a very small number of the analyzed cells can successfully reach a pluripotent state, suggesting many of the events observed are common to both cells that will become iPSCs and those that will fail. Even partially reprogrammed, so-called, pre-iPS (piPS) cells give rise to fully reprogrammed cells with a very low frequency and it is not clear if these piPS cells are on the correct route to achieve a fully reprogrammed state. Thus there is still a lack of knowledge as to how successful reprogramming occurs and what route cells take in order to achieve a pluripotent state. To address this question, we have identified novel REProgramming cell Surface markers (REPS1 and REPS2) whose expression dynamically changes during reprogramming using a highly efficient secondary reprogramming system. These REPS markers, together with a Nanog-GFP reporter, have enabled the monitoring of changes in cellular populations during reprogramming, and to isolate distinct populations that are in different stages of the reprogramming process. Interestingly, some, but not all, cells that become iPSCs start expressing Nanog-GFP reporter before gaining iPSC-like REPS expression. Cells in different stages displayed different iPSC-forming potencies as well as different pluripotent gene expression profiles. These data indicates reprogramming progresses in a stepwise manner and there may be more than one main route to an iPS cell state. Our unique reprogramming system and cell surface markers will allow further investigation of the molecular mechanisms of successful reprogramming.

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Poster Board Number: F-3232

GENERATION OF INTEGRATION-FREE MOUSE AND HUMAN IPS CELLS USING HUMAN ARTIFICIAL CHROMOSOMES

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Induced pluripotent stem cells (iPSCs) have great potential in regenerative medicine. However, in order to achieve clinical application, generating virus-free and integration-free iPSCs is crucial. During the recent several years, various such methods have been reported, but further improvements remain to be required, such as quality of individual iPS cells, reprogramming efficiency and genome integrity. Human artificial chromosomes (HACs) have unique characteristics as gene-delivery vectors, including episomal transmission and transfer of multiple, large transgenes. Furthermore, inserting all expression constructs into a defined cloning site on the HAC vector, which was maintained stably and independently of host chromosomes in cells, resulted in relatively homogenous expression levels of the transgenes in HAC donor and recipient cells. Once configuration of an expression cassette in the HAC vector is optimized for the generation of iPS cells, the resulting uniformity of transgene expression in target cells is an advantage in promoting the reprogramming efficiency and reducing the clonal variation in the resulting iPS cells. Thus, the HAC-based reprogramming strategies are expected to be more effective in establishing homogenous iPS clones than other methods, including DNA transfection and viral transduction, which are both unable to regulate the quantity of xeno-products in modified cells. Here, we demonstrate that a HAC vector containing expression cassettes for four reprogramming factors and a p53-knockdown construct (iHAC2) efficiently reprogrammed mouse embryonic fibroblasts (MEFs) to pluripotency. In addition, iPS clones established by iHAC2 exhibited relatively uniform pluripotency, and integration-free iPS cells were obtained from these reprogrammed cells. Next, we constructed another HAC vector for reprogramming human somatic cells by modifying iHAC2 with human P53 knockdown construct in addition to multi copies of four reprogramming factors (iHAC-hP53). The iHAC-hP53 could reprogram human fibroblast (HFL-1), but only partially. Therefore, we construct HACs with further reprogramming factors to optimize the construction for reprogramming human cells, and compare the reprogramming ability among the HAC constructs.

Poster Board Number: F-3233

IMMUNOGENIC POTENTIAL OF MOUSE PLURIPOTENT STEM CELLS

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Reprogramming of somatic cells into pluripotent stem cells has been reported by introducing a combination of several transcription factors (Oct3/4, Sox2, Klf4, and c-Myc). The induced pluripotent stem (iPS) cells from patient's somatic cells would be useful source for drug discovery and cell transplantation therapies. One advantage of iPS cells is the potential usage of autologous and HLA-matched transplantation. A study raised question about this point. Zhao and his colleague showed immunogenicity of mouse iPS cells in 2011. However there should be some discussion about the immunogenicity of iPS cells, as they used undifferentiated iPS cells for transplantation which would never happen in medical transplantation. We have established several clones of mouse ES and iPS cells from C57BL/6 mice, and planning to carry out the transplantation after neural differentiation. In this meeting, we would like to discuss the progress of the study.

Poster Board Number: F-3234

INDUCED PLURIPOTENT STEM CELLS PRESENT THE CYTOPROTECTIVE EFFECT IN THIOACETAMIDE-INDUCED ACUTE HEPATIC FAILURE MICE

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Acute or fulminant hepatic failure (AHF) is a severe liver injury accompanied by hepatic encephalopathy which causes multiorgan failure with a high mortality rate. Liver transplantation is an effective treatment for various end-stage liver diseases. However, the shortage of donor organs and complications associated with rejection hinder the organ transplantation. Inducible pluripotent stem (iPS) cell is a kind of novel stem cell population induced from adult somatic cells by transduction of defined transcription factors. The differentiation potentials of iPS cells have been demonstrated by different laboratories. We investigated whether iPS cells transplantation could improve the physiological condition in the mouse model of liver injury. Acute/fulminant hepatic failure (AHF) was induced by injection of thioacetamide (TAA) into mice. Then, iPS cells were injected via tail vein into mice with normal or injured livers. Herein we demonstrated an efficient method to differentiate iPS cells into hepatocyte-like cells that expressed hepatic markers, including albumin, α-fetoprotein, and hepatocyte nuclear factor-3β, and exhibited hepatic functions. Furthermore, injected iPS cells were able to engraft a recipient liver and rescue TAA-induced liver failure. We found that the motor activity and liver biochemistry of iPS-treated mice were dramatically improved compared to those of control groups. Histological results also showed that the numbers of infiltrative lymphocyte were obviously reduced 72 hours post the iPS treatment. The Hoechst labeled-iPS cells were easily observed by fluorescent microscopy in injured livers but not normal livers. The data suggest using iPS cells to overcome these issues while a large number of iPS cells can be prepared *in vitro*. Our results also support that iPS cells could immediately improve liver functions shortly after transplantation.

Poster Board Number: F-3235

EFFECTIVE SELECTION OF MOUSE INDUCED PLURIPOTENT STEM CELLS VIA DOX-INDUCIBLE LENTIVIRUS SYSTEM

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Induced pluripotent stem (iPS) cell can be a valuable cell source for drug screening and toxicity assessment in pharmacology and Toxicology. To establish somatic reprogramming system, firstly induction of iPS cells from mouse embryonic fibroblast (MEF) was conducted by doxycycline-inducible lentiviral infection. After introduction of four reprogramming factors (Oct4, Sox2, Klf4 and

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c-Myc), morphological change was observed at day 3 and ES-like colonies were emerged at 2 weeks under doxycycline treatment (Dox+). After Dox treatment (Dox+), 28 pre-iPS clones out of total 104 colonies were selected by AP staining. The selected subclones showed different growth pattern after extended culture; 5 clones (#15, #60, #68, #91 and #92) grew morphologically like ES cells while 23 clones showed morphologically heterogenous clusters after subculture. Five clones still maintained ES-like shape while 23 clones differentiated wholly fibroblast-like shape having small portion of ES-like colonies without Dox treatment. It suggested that Dox-inducible reprogramming technique could be rapid and effective system for sorting fully reprogrammed or partially reprogrammed iPS without marker gene expression (Nanog-, Oct-GFP and Oct-Neo). To identify reprogrammed status of selected iPS clones, total 8 pre-iPS clones including four (#15, #60, #68 and #91) maintained without Dox, two (#89 and #102) grown with Dox and two (#89D and #102D) derived from #89 and #102 subscultured without Dox were characterized their pluripotent nature; expression of ES-specific markers, gene expression, DNA methylation status, in vitro and *in vivo* differentiation. AP-positive 6 clones were strongly reacted against SSEA-1, Nanog and Oct4 antibody and expressed Nanog, stella, rex1, Oct4, Sox2, Klf4 and c-Myc. However, #89D and #102D represented weak and partial expression of Nanog protein regardless of strong Nanog expression. DNA methylation of five clones (#15, #60, #68, #91 and #89) were demethylated similar with that of ES cells but #102 clone showed intermediated methylation status between ES and MEF. However, #89D and #102D showed hypermathylated in CpG site of Nanog and Oct4 promoter like MEF methlyation pattern. In in vitro differentiation, all clones except for #89D and #102D formed embryoid bodies and they were reacted with nestin, SM22-alpha and Sox17 which are representing ectoderm, mesoderm and endoderm marker, respectively. The present study suggested that fully reprogrammed iPS cells were not affected by the expression of exogenous genes while partially reprogrammed iPS cells were only maintained by Dox-induced exogenous 4 factors. In conclusion, fully reprogrammed mouse iPS cells were easily and effectively selected by Dox-inducible lentiviral system and partially reprogrammed mouse iPS cells also could be valuable cell sources to understand later reprogramming status.

Poster Board Number: F-3236

INHIBITION OF ENDOGENOUS TGF-B SIGNALS ENHANCES THE EFFICIENCY OF THE GENERATION OF INDUCED PLURIPOTENT STEM CELLS FROM MOUSE EMBRYONIC FIBROBLASTS THROUGH INDUCING MESENCHYMAL-TO-EPITHELIAL TRANSITION

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The combined activity of a defined set of transcription factors (Oct4, Sox2, Klf4 and c-Myc) can reprogram somatic cells into induced pluripotent stem (iPS) cells. However, concerns raised by the current reprogramming protocols involving the delivery of transgenes have prompted researchers to identify chemical compounds including the inhibitors of signals mediated by transforming growth factor (TGF)-β as inducers of reprogramming. Several lines of evidence have revealed that mesenchymal-to-epithelial transition (MET) takes place during the initial phase of reprogramming. While TGF-β induces epithelial-to-mesenchymal transition (EMT), it is not fully elucidated whether MET is induced by inhibition of endogenous TGF-β signals in fibroblasts. In order to study the molecular mechanisms how inhibition of TGF-β signals enhances the efficiency of reprogramming, we examined the effects of RepSox, a small molecule compound that inhibits the TGF-β type I receptor kinase, on the MET and reprogramming of mouse embryonic fibroblasts (MEF). We first examined which reprogramming factors induce MET by retrovirally introducing Oct-4, Sox2 or Klf4 to MEF. Only Klf4 was able to elevate the expression of epithelial markers including E-cadherin and claudin-11 and decrease the expression of mesenchymal markers including α-smooth muscle actin. Addition of RepSox to the culture of MEF significantly induced MET. Since Klf4 plays central roles in the MET during reprogramming, we examined whether inhibition of TGF-β signals may replace Klf4 during reprogramming. While introduction of Oct-4, Sox2 and c-Myc was not capable of reprogramming MEF, addition of RepSox to the culture of MEF infected with retroviruses encoding Oct-4, Sox2 and c-Myc induced the generation of iPS cells. Although a previous report showed that the effect of RepSox was evident during the maturation phase of reprogramming, we found that RepSox significantly elevated the efficiency of reprogramming when added during the initial phase in which MET takes place. These results suggest that inhibition of TGF-β signals regulates the reprogramming via multiple steps of reprogramming of fibroblasts into iPS cells.

Poster Board Number: F-3237

GRAFTED UNSAFE HUMAN IPSC-DERIVED NEUROSPHERES PROMOTE TEMPORAL FUNCTIONAL RECOVERY IN SPINAL CORD INJURED MICE, FOLLOWED BY NEOPLASM FORMATION AND MOTOR DETERIORATION AFTER LONG TERM OBSERVATION

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Induced pluripotent stem cells (iPSCs) have the potential to resolve the ethical issues and immunological rejection associated with embryonic stem cells (ESCs). Recently, we have reported the effectiveness of transplantation of safe human iPSC (201B7)-derived neurospheres (hiPSC-NSs) for spinal cord injury (SCI) in NOD-SCID mice (Nori et al., PNAS 2011). In the present study, we performed transplantation of unsafe hiPSC (253G1)-NSs into injured spinal cord of NOD-SCID mice to examine their risks as a cell source of transplantation. We performed neural differentiation of hiPSCs, using the cell line, 253G1, generated from adult human dermal fibroblast by retroviral transduction of three transcription factors (Oct3/4, Sox2 and Klf4). 253G1-NSs were mainly differentiated into neurons *in vitro*. Adult female NOD-SCID mice were used in this study. Contusive SCI was induced at Th10 level using IH impactor. Nine days after injury, mice were to receive randomized 253G1-NSs (n=32) or PBS (n=26). Motor functions had been assessed until 56 days after SCI by BMS, Rotarod test and Treadmill gait analysis using DigiGait system, followed by histological analysis. To investigate the long-term safety of the grafted 253G1-NSs, we extended the follow-up period of some mice (253G1-NS group (n=22) and PBS group (n=16)) to 112 days after SCI. At 56 days after SCI, grafted 253G1-NSs survived and differentiated into three neural lineages in the injured spinal cord. We found that most 253G1-derived neurons were GABAergic, and formed synapses with host-mouse neurons. Grafted 253G1-NSs also promoted axonal regrowth and angiogenesis. Consistently, RT-PCR revealed high expression of trophic

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factors (BDNF, NGF, HGF and VEGF) derived from grafted human cells as well as host mouse cells. As a result of these positive effects, 253G1-NS group exhibited significantly better functional recovery than the PBS group. At 56 days after SCI, there was no evidence of mass formation in 253G1-NS group. It is important to address safety issues about transplantation of hiPSC-NSs after long term observation. While motor functional recovery was once observed in 253G1-NS group until 56 days after SCI, they showed gradual deterioration of function at 63 days and thereafter. By histological analysis at 112 days after SCI, 253G1-NSs grafted mice showed neoplasm formation. These neoplasms consist of Nestin+ microcystic mass. Notably, the content of Nestin+ cells and Ki-67+ cells increased from 56 days to 112 days after SCI. We also observed correlation between the content of Nestin+ cells as well as the content of Ki-67+ cells and the diameters of neoplasms. Moreover, we evaluated mRNA expression of human OCT3/4, KLF4, MYC and NANOG 14 days and 112 days after SCI. At 112 days after SCI, the expression of OCT3/4 was up-regulated, while the other factors were not. It suggested that the up-regulated expression of OCT3/4 related to neoplasm formation after transplantation. The present study demonstrates even unsafe hiPSC-NSs could have temporal potential benefits for SCI. However, subsequent long term observation is required for the clinical application of hiPSC-NSs, because unsafe hiPSC-NSs formed neoplasms after long period of time.

Poster Board Number: F-3238

GENERATION OF HIRCINE INDUCED PLURIPOTENT STEM CELLS BY SOMATIC CELL REPROGRAMMING

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Goat embryonic stem cell lines would be useful for generating precise gene-modified goat. To date, many unsuccessful efforts have been made to derive goat embryonic stem cells from early embryos. Here, we report that goat somatic cells could be directly reprogrammed to pluripotency using eight defined factors. Our observations indicated that goat somatic cells are more difficult to be reprogrammed than somatic cells from other species. We demonstrated that goat iPS cells expressed embryonic stem cell markers, including alkaline phosphatase, Oct3/4, Nanog, Sox2, Rex1, SSEA-1, Tra-1-60, Tra-1-81 and CDH1. Goat iPS cells exhibited a normal karyotype, and were able to differentiate into all three primary germ layers both in embryoid bodies and in teratomas. Our study may help to reveal the properties of goat pluripotent stem cells and provide a system to screen the culture conditions for goat ES cells. Moreover, goat iPS cells may be directly used in generating precise gene-modified goat.

Poster Board Number: F-3239

EROSION OF DOSAGE COMPENSATION IMPACTS HUMAN IPS CELL DISEASE MODELING

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Considerable epigenetic variation has been observed among individual human induced pluripotent stem (hiPSC) lines. However, it is currently unclear how this variation influences the use of hiPSC in disease modeling. Here, we have investigated the impact of changing patterns of X-chromosome inactivation (XCI) on the modeling of Lesch-Nyhan Syndrome (LNS), which is caused by mutations in the X-linked HPRT gene. We demonstrate that HPRT null male hiPSC lines can be used to produce a new robust model system for the study of LNS. When male hiPSCs (HPRT-/Y) were differentiated into neurons, they reproducibly generated fewer neurons with shorter neurites. We further show that these neuronal defects are cell autonomous and can be rescued by exogenous expression of functional HPRT. Our results confirm that early passage female hiPSC lines contained an inactive X-chromosome, and that this inactive chromosome was in every case the same inactive X-chromosome found in the somatic fibroblast that they were derived from. Therefore, low passage female heterozygous cell lines carrying the same HPRT mutation on the active X-chromosome (XaHPRT- / XiHPRT+) generated identical LNS neuronal phenotypes to those seen using male lines. However, as we subjected these hiPSCs to long-term culture, we found that female hiPSC lines lost cytological signs of XCI, and that these events coincided with ectopic reactivation of the functional HPRT gene from the inactive X. We further show that this "erosion" of dosage compensation could not be reversed by either differentiation or by further reprogramming. Consequently, female lines that have lost XCI marks no longer exhibited the LNS disease phenotypes when differentiated into neurons. Through multiple lines of evidence, including functional assays of HPRT activity and analysis of X-chromosome-wide levels of DNA methylation and transcription, we show that erosion of dosage compensation results in the de-repression of most genes normally subjected to X-inactivation and that this erosion had already occurred in the majority of female pluripotent stem cell lines. In summary, the erosion in dosage compensation we have observed leads to bi-allelic expression of X-linked genes in stem cells and their differentiated derivatives and thus should be closely monitored as it can substantially interfere with the interpretation of *in vitro* models of human diseases.

Poster Board Number: F-3240

ALLOGENEIC TRANSPLANTATION OF INDUCED PLURIPOTENT STEM CELLS IN A PORCINE MODEL OF CHRONIC MYOCARDIAL ISCHEMIA FAILED TO STIMULATE MYOCYTE DIFFERENTIATION

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Background. Recently, pig induced pluripotent stem cells (iPSCs) have been produced by by ectopic expression of four human transcriptional factors, OCT4, SOX2, KLF4, and cMYC in pig fetal fibroblasts through a lentiviral vector. The pig iPSCs showed pluripotency via in vitro differentiation assays and teratoma testing. However, these cells have failed to differentiate into beating myocytes in vitro. To determine whether *in vivo* conditions would drive iPSC differentiation into myocytes, we performed allogeneic transplantation of the pig iPSCs into areas of chronically ischemic myocardium. Methods. Pig iPSCs were expanded by standard ES cell culture method, and allogeneically transplanted into chronically ischemic myocardium of eight Yorkshire pigs by direct intramyocardial injection (four 10-cm culture dishes, total of 10-16 million cells, suspended in 2.5 ml of saline, with 25 injection sites). Cohorts of two animals were sacrificed at 2, 4, 8 wks and 3 months after injection to study the differentiation of the injected cells. Results. After transplantation, there were no signs of adverse side effects or graft versus host disease at any time point. Two weeks after injection, clusters of SSEA-4 positive cells were detected in the frozen sections of the injected area using immunofluorescent staining. Four to 8 weeks later, these clusters of cells started to proliferate, within the injected area, into spherical shaped small

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tumors surrounded by thin capsules. The cells inside these tumors showed homogeneous phenotype with no signs of differentiation into any lineage. Three months after the injection, no such small tumors were found; instead, similar SSEA-4 positive cell clusters were found in the injected area but in smaller numbers. By immunofluorescent staining, few smooth muscle actin or vWF positive cells were found inside the cell clusters, but no positive desmin cells were found, and by RT-PCR, the expression of VEGF, FGF, ANRT were significantly higher in the injected myocardium compared with none injected, suggesting that injected iPSCs were not differentiated into myocytes and might contribute to the formation of some new vessels. Conclusions. Allogeneic transplantation of iPSCs can be safely used to test the lineage differentiation of stem cells for the future iPSC-based therapy. Despite an ischemic environment, pig iPSCs continue to proliferate *in vivo* for three months after injection. However the proliferation ability of the iPSCs was limited within the immunoconpetent hosts. For future cell-based therapy, iPSCs with pro-myocyte differentiation will be needed to study their potential for myocardial regeneration.

Poster Board Number: F-3241

MOLECULAR EVALUATION OF AN INTEGRATION-FREE IPSC BASED MODEL OF ALZHEIMER'S DISEASE IN THE INDIAN COHORT

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Alzheimer's disease (AD) is a neurodegenerative disorder, characterized by cognitive impairment, neuronal loss in the limbic and neocortex region and formation of amyloid plaques and neurofibrillary tangles. In India, AD is estimated to have a lower prevalence (3-9%) than the developed countries (12-24%), but due to the large population and the recent increase in longevity, this number is estimated to rise to 6.5 million people by 2025 from the present statistic of 3.6 million. Thus, AD will become a significant burden of disease in India. Further, molecular characterization of well-characterized AD pedigrees from this cohort suggests population specific causal and/or modifier genes. Detailed evaluation of these is warranted for better understanding of the underlying disease mechanism. The biggest limitation of AD research has been the lack of suitable models (both in vitro and *in vivo*) that mimic the complex disease process. Induced pluripotent stem cells (iPSCs) offer an interesting tool since they are genetically identical to patients presenting the disease and can be differentiated in vitro to specific neuronal cell types. The most immediate value of hiPSC lines from patients carrying either rare mutations or common risk alleles is that it allows researchers to potentially examine mechanisms underlying pathogenesis. These cells can be grown for an extended period in culture facilitating detailed in- vitro interrogation of the disease in multiple ways. The utility of this system thus, depends heavily on the development of assay systems that can recapitulate the *in vivo* disease condition. Reprogramming of somatic cells to pluripotency is achieved by the expression of certain transcription factors and in the recent past several gene delivery systems have been employed to achieve this. To our knowledge, most of the patient specific lines reported so far are derived using viral transduction and this may affect the phenotype under investigation. We have used an integration-free plasmid-based method and report successful generation of hiPSCs from AD patients and normal controls using patient derived lymphoblastoid cell lines (LCLs) as the source tissue. Previous reports have used fibroblasts to generate iPSCs from AD patients. LCLs offer advantages as it is much less invasive to obtain peripheral blood, easier to establish lines from and they also do not undergo genomic alterations for a large number of passages. Fully transformed adherent hiPSC colonies were obtained within 20 days from the non-adherent LCLs. These colonies were cultured for a number of passages to obtain stable clones which were characterized for self-renewal and differentiation properties. We are currently evaluating these lines for disease specific phenotypes using cell-based assays, in conjunction with information from genetic analysis. Most if not all the genetic and molecular data on AD published to date is in the European cohort. We need to know more about risk factors in non-European populations as that will improve our understanding of the disease mechanism. These patient-specific iPSC lines will serve as good in-vitro models to study molecular players involved in disease pathology.

Poster Board Number: F-3242

MODELING INHERITED SKELETAL DISEASE IN HUMAN IPS CELLS

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Muscloskeletal diseases are a growing medical concern worldwide. Unfortunately, management for these diseases remains rudimentary due to a lack of robust human models. Skeletal tissues derived from induced pluripotent stem (iPS) cells provide promising tools for studying human genetic diseases from patients with known phenotypes. However, our understanding of how pluripotent stem cells can be guided to form skeletal tissues is rudimentary. We established a robust and simple *in vitro* model of osteoblast differentiation based on human iPS cell cultures. Osteogenic medium containing FBS, ascorbic acid, dexamethasone, and beta glycerol phosphate promoted iPS cell differentiation into osteoblasts and strong mineralization after 15 days of culture as detected by von Kossa staining. We also found high expression of osterix at day 6 of differentiation and increasing levels of collagen I and osteocalcin at days 15 and 24, indicating formation of both immature and mature osteoblasts in our culture system. To discern whether the iPS model could recapitulate aspects of human skeletal disease, we established human iPS cell lines from patients with Fibrodysplasia Ossificans Progressiva (FOP), a disease with dramatic increases in skeletal tissue formation. Multiple iPS cell lines were successfully generated using viral transduction of four pluripotency-inducing factors (Oct4, Sox2, Klf4, and cMyc) into skin fibroblasts from two independent FOP patients carrying the classical R206H mutation in ACVR1. We confirmed that the FOP iPS cells retained the R206H ACVR1 mutation and expressed pluripotency markers. Embryoid bodies derived from FOP iPS cells expressed marker genes for all three germ layers. Osteogenic differentiation of the FOP iPS cells showed significantly more robust mineralization with an earlier onset as compared to wildtype controls. Our results indicate that the R206H ACVR1 mutation in FOP iPS cells may confer increased mineralization activity as compared to control iPS cells. These findings identify a potential late effect of the genetic mutation in the development of FOP heterotopic ossification lesions and demonstrate that stem cell-based models could be valuable for studying genetic diseases of osteoblast formation and function. These models will facilitate

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dissection of the molecular pathogenesis of human skeletal disease and facilitate the identification of new therapeutic approaches for skeletal disorders.

Poster Board Number: F-3243

DOWN SYNDROME INDUCED PLURIPOTENT STEM CELLS HAVE MORE HAEMATOPOIETIC POTENTIAL

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Down Syndrome (DS) children have a high risk of developing leukaemia. The blood disorder starts in the fetus, presenting as Transient Abnormal Myelopoiesis (TAM) in neonates which, in some cases, evolves into leukaemia, particularly Acute Megarkaryoblastic leukaemia (AMKL). In this study we aim to use iPSCs made from DS (Tri21) fibroblasts as a novel in vitro model to elucidate the abnormal haematopoiesis observed *in vivo*. Stable iPSC lines were created from normal and DS fibroblasts and extensively characterised to be like embryonic stem cells. iPSC derived embryoid bodies (EBs) were then cultured in defined growth medium (SCF, BMP4, FLT3 ligand, VEGF, TPO) to promote haematopoietic differentiation. Flow cytometry detected CD34+ cells (5-15%) after about 10 days. More mature myeloid cells (CD45+/CD33+) were also detectable and increased to over 30% after 2 weeks. In these cultures, we detected a greater percentage of haemogenic KDR (VEFDR2)+ cells derived from Tri21-iPSCs compared to control iPSCs (2.5% vs 0.3%). The HSCs derived from Tri21-iPSCs also had more clonogenic potential than those from the normal counterpart in semi-solid culture assays. A spectrum of colonies were detected although we noted more CFU-E, BFU-E and CFU-GEMM colonies generated from Tri21-iPSCS compared to control cells. Overall, our results indicate that the in vitro haematopoiesis of Tri21 iPSCs reproduces some of the characteristics of the aberrant myeloproliferation seen in children and in mouse models bearing Tri21. These iPSCs therefore provide an in vitro human model to evaluate steps involved in the evolution of DS-AMKL. Gene expression profiling of isolated progenitors aiming to identify abnormal gene expressions linked to the aberrant haematopoiesis of Tri21-iPSCs will be discussed.

Poster Board Number: F-3244

GENERATION OF INDUCED PLURIPOTENT STEM CELLS FROM WERNER SYNDROME PATIENTS

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Werner syndrome (WS) is a rare autosomal recessive disorder characterized by premature aging, chromosomal instability and cancer predisposition. Fibroblasts derived from WS patients exhibit premature replicative senescence due to telomere dysfunction. Although WRN, a gene responsible for the disease, and encoding a DNA helicase was identified fifteen years ago, and function of a protein encoded by the gene had been extensively studied, the pathogenesis of Werner syndrome is remains to be elucidated. Somatic cells can be reprogrammed into induced pluripotent stem (iPS) cells by ectopic expression of transcription factors, such as Oct4, Sox2, Klf-4 and c-myc. iPS cells are expected to be useful for many applications in medicine as well as in basic research, because of their potential to differentiate into all the cells composing the body of an individual, and ability to proliferate indefinitely. To develop a new therapeutic strategy for the treatment of WS, reveal the pathogenesis of WS and provide drug screening tools for WS, we have attempted to generate iPS cells from fibroblasts of WS patients by retroviral expression of Yamanaka four factors. WS iPS colonies showed similar morphologies to colonies of normal iPS as well as of human ES cells, and exhibited alkaline phosphatase activity. WS iPS cells expressed not only pluripotent genes such as Nanog, Rex1 and DNMT3b in addition to endogenous Oct4 and Sox2 genes, but also an immortalizing gene, telomerase catalytic subunit hTERT. The cumulative passages of WS iPS cells are proceeding for almost two years, supporting the notion that induction of telomerase activity during reprogramming endow parental cells of WS patients with indefinite proliferative potential, and overcome their premature senescence phenotype. These iPS cells will provide opportunities with us to elucidate the pathogenesis and to develop regenerative medicine for WS patients.

Poster Board Number: F-3245

SMALL-SCALE, FOOTPRINT-FREE GENERATION OF HUMAN IPSC FROM CELL REPOSITORY SPECIMENS TO STUDY NEUROPSYCHIATRIC AND ADDICTION DISORDERS

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Embryonic stem cells cannot be used to study certain types of addiction disorders and other psychiatric diseases, particularly those that do not appear until late in life. Until recently, cellular surrogates, such as blood or fibroblasts, have been the only available cell types in which to study the genetic mechanism of these diseases. These surrogates are often poor models of the disease since the disorders are mainly manifested in central nervous system (CNS) neurons. A promising approach to this problem is to differentiate iPSC derived from clinically affected subjects into neurons. Although iPSC were initially derived from skin fibroblasts, many other cell types have now been successfully reprogrammed. The use of blood to generate iPSC is ideal as the collection of blood is routine as well as less invasive than the collection of skin fibroblasts through biopsy. Additionally many cell repositories, including the National Institute of Mental Health's Center for Collaborative Genomic Studies on Mental Disorders, store blood cells, but until recently have not stored skin fibroblasts. A major advantage is that the genomes of these existing blood samples have been characterized (e.g., a million SNPs) and clinical data on these subject samples is accessible to investigators. Using cryopreserved lymphocytes (CPLs) from subjects that had been clinically diagnosed with addiction disorders and have been genetically identified to contain mutations in either the CHRNA5 or OPRM1 genes, we have produced iPSC by delivering the Oct4, Sox2 and Klf4 and c-Myc transcription factors via Sendai virus. Since Sendai virus delivery is non-integrating, there is no risk of insertional mutagenesis from the reprogramming and the resulting iPSC are "footprint-free." By generating, expanding and culturing the iPSC in 96 well plates, we have increased the throughput of our experiments allowing us to rapidly generate 16 iPSC lines that contain these mutations. Once generated the iPSC

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are subjected to standard neurogenesis protocols and are being used to study the mutated signalling pathways that are believed to underlie certain addiction disorders. The combination of well-characterized repository specimens, footprint-free reprogramming, and high-throughput methods provides an example of generating valuable cellular tools for studying the causes and treatment of addiction and neuropsychiatric disorders.

Poster Board Number: F-3246

MULTIPLEXED HIGH CONTENT ASSAYS FOR PREDICATIVE HEPATOTOXICITY USING INDUCED PLURIPOTENT STEM CELL DERIVED HEPATOCYTES.

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A large percentage of drugs fail in clinical studies, or are withdrawn from the market due to hepatic toxicity. Therefore development of highly predicative *in vitro* assays suitable for safety and efficacy testing is extremely important for improving the drug development process and reducing drug attrition. Accordingly, there is great interest in using stem cells as tools for screening compounds during early drug development. Human hepatocytes derived from stem cell sources can greatly accelerate the development of new chemical entities and improve drug safety by offering more clinically relevant cell-based models than those presently available. Human induced pluripotent stem cell (iPSC) derived hepatocytes express appropriate hepatocyte markers and demonstrate intrinsic hepatocyte functions similar to primary cells. We demonstrate two models for assessing general and specific hepatotoxicity that are suited for automated screening environments. One model assesses toxic effects of compounds by measuring hepatocyte viability and intrinsic hepatocyte functions. Multi-parametric read-outs allow simultaneous assessment of viability, membrane permeability, , lipid accumulation, and cytoskeleton integrity. Another method measures mitochondrial depolarization in live cells, which has been shown to be an early signal for hypoxic damage or oxidative stress. Various image processing modules, including multi-wavelength cell scoring, live-dead and granularity provide multiple outputs characterizing phenotypic changes. Tools for cluster and principal component analysis provide additional insights into assay results. Assays will be applicable for assessment of potential hepatotoxic effects and for development of cell-based models of diseases. We demonstrate the utility of assays using several kinase inhibitors, anti-inflammatory, and anti-cancer compounds.

Poster Board Number: F-3247

MODELING GASTROINTESTINAL AND LIVER GENETIC DISEASES USING INDUCED PLURIPOTENT STEM CELLS

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Patient-specific induced pluripotent stem cells (iPSC) have attracted considerable attention as an alternative to embryonic stem cells for disease modeling, drug screening and regenerative medicine. In this study we employed our recently developed STEMCCA lentiviral reprogramming vector to derive iPSC from patients with Hereditary Hemocromatosis (HH) and Familial Adenomatous Polyposis (FAP). These iPSC expressed the pluripotency markers SSEA-4, Tra-1-60 and Tra-1-81 and displayed a normal karyotype. Furthermore, HH-iPSC were capable of efficient differentiation into c-kit+/CXCR4+ endoderm cells that could be specified into hepatocyte progenitors expressing albumin, AFP and HNF4a through the addition of soluble factors known to promote liver development *in vivo*. Importantly, these cells showed upregulation of the HH-related genes HFE and hepcidin during the last stage of differentiation, providing a bona fide experimental platform to study the molecular basis of the liver disease in vitro. A similar approach has been taken to model FAP, via directed differentiation of FAP-disease specific iPSC into intestinal epithelial progenitor cells using chemically defined conditions. These cellular platforms in combination with the use of novel gene correction methodologies to obtain patient-specific genetically corrected iPSC open a new era in disease modeling and cell replacement therapies.

Poster Board Number: F-3248

GENERATION OF MENKES DISEASE INDUCED PLURIPOTENT STEM CELLS

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Copper is an essential trace element for all the living organisms, functioning as an integral part of cuproenzymes such as lysyl oxidase, dopamine β-oxidase, cytochrome C oxidase, and tyrosinase. Thus, tight regulation of copper homeostasis which is maintained by copper uptake, transport, storage and excretion is required for cell survival. Menkes disease is a representative genetic disease related with disruption of copper homeostasis caused by mutated P-type ATPase copper-transporting ATPase 1(ATP7A) gene. Menkes disease is characterized by infantile-onset neurodegeneration, failure to thrive, coarse hair, and connective tissue abnormalities. Although various clinical symptoms are related with dysfunction of cuproenzyme, exact pathophysiological mechanisms are still poorly understood. In this study, fibroblasts of Menkes disease patients carrying ATP7A gene mutation(c.4005+5G>A) could be normally reprogrammed to the induced pluripotent stem (iPS) cell by ectopic expression of Yamanaka's factors. Menkes disease-iPS cells had normal karyotype and expressed endogenous pluripotency marker genes at transcription and protein levels. The Menkes disease-iPS cells can be used as a cellular model for studying pathophysiology related with copper homeostasis.

Poster Board Number: F-3249

MODELING PLATELET DEFECTS OF WISKOTT-ALDRICH SYNDROME PATIENTS USING INDUCED PLURIPOTENT STEM CELLS

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Induced pluripotent stem cells(iPSCs) hold great promise for the development of patient-specific cell therapies and also provide a platform for modeling human diseases in vitro. Since human biology is fundamentally different from other species, iPSC models can be used for validating what learned from animal models. Wiskott-Aldrich syndrome (WAS) is a severe X-linked recessive disorder that is characterized by, microthrompocytopenia, complex immunodeficiency, and increased risk in developing autoimmune diseases and hematologic malignancies. WAS is caused by the mutation in

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gene encoding Wiskott-Aldrich syndrome protein (WASP) which play an important role in the organization of actin cytoskeleton and chromatin structure of hematopoietic cells. The pathophysiology of microthrompocytopenia and autoimmunity in WAS are not well understood. In contrast to human patients, murine models of WASp deficiency exhibit only mild thrombocytopenia, and platelets are of normal size. Moreover, WASp deficiency mice do not develop eczema, hematopoietic malignancies. To further elucidate the role of WASP in the development and functions of hematopoietic cells, we first generated several lines of induced pluripotent stem cells from dermal fibroblasts of WAS patients. When WAS iPSCs were differentiated into hematopoitic progenitor and platelets via ES-sacs according to protocols by Takayama et al., 2007, 2010, we found that while the ability to generate primitive hematopietic progenitors were largely indistinguishable between WAS iPSCs, normal iPSCs and 2 ES cell lines, WAS iPSCs produced much less number of platelets than control. Platelets generated from WAS iPSCs are significantly smaller similar to what observed in WAS patients. Electron microscopic study demonstrated that WAS iPSCs exhibited defects at pro-platelet formation when co-cultured with OP9 stromal cells. Overexpression of WASp in WAS iPSCs using lentivirus vector reduced the defects observed. We are currently investigating the role of WASp in early lymphogenesis using tet-on WAS- WAS iPSCs. Since current available techniques for *ex vivo* expansion of long-term hematopoietic stem cells still need improvement, it is not possible to significantly expand clonally-selected hematopoietic stem cells from WAS patient in vitro, WAS iPSCs could prove to be a useful tool for studying disease pathophysiology. With advancement in techniques for safer iPS cells generation and genome editing, disease-corrected WAS iPS cells has a potential to become a source of autologous cell therapy in the future.

Poster Board Number: F-3250

GENERATION OF TRANSGENE-FREE HUMAN INDUCED PLURIPOTENT STEM CELLS FROM THE PATIENTS WITH INTRACTABLE DISEASES

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Induced pluripotent stem cells (iPS cells) derived from patients with intractable diseases represent a powerful tool not only for biomedical research but also for investigating the effects of drugs on patient-derived somatic cells. However, in many intractable diseases, the number of cases is restricted, and biological samples including blood, tissue and biopsy are very few. In addition, another limitation that the integration of viral transgenes carrying reprogramming factors into the host genome that includes the risk of tumorigenicity has remain uncleared. Thus, transgene-free disease-derived iPS cells are required for generating the disease model which properly reflects pathogenetic features. To generate transgene-free disease-derived iPS cells, we have employed Sendai virus (SeV) vectors. Because SeV vectors replicate in the form of negative-sense single-stranded RNA in the cytoplasm of infected cells, and do not integrate into the host genome. In addition, SeV vectors are able to be removed by repeatitive passages during the culture. Thus, the resulting iPS cells is genetically intact and is suitable for detailed analysis of the disease-specific iPS cells. Here, we present some cases of disease-derived iPS cells generated. In our project, various kinds of intractable disease-derived iPS cells including metabolic disorder, neurodegenerative disease and etc. were generated from the patients' skin fibroblasts by SeV vector. Overall, these transgene-free disease-derived iPS cells we generated are expected to be applicable to disease study and drug discovery applications.

Poster Board Number: F-3251

INDUCED PLURIPOTENT STEM CELLS BASED MODEL SYSTEM FOR STUDYING HUNTINGTON'S DISEASE.

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Discovered in 2006, induced pluripotent stem cells (iPSCs) have properties similar to embryonic stem cells (ESCs) and can be derived from somatic cells of the adult organism. There are two main areas of practical application of iPSCs technology: regenerative medicine and human disease modeling. Neurodegenerative disorders of central nervous system are of particular interest for the disease modeling due to the limited access to the cells of human brain for research applications or the lack of adequacy of existing animal and cell models. Disease model based on iPSCs-derived neurons of patients with Huntington's disease (HD) may become valuable instrument to study HD and to perform drug screening. In our study we generated a number of human iPSC lines from HD patients. Human dermal fibroblasts were obtained from skin biopsies and triplet amplifications in huntingtin gene were determined by PCR analysis. To generate iPSC lines fibroblasts were infected with lentiviral constructs encoding transcription factors: OCT4, SOX2, c-Myc, KLF4. We demonstrated that iPSC lines expressed pluripotent cells markers and were able to differentiate into the cells derivatives of three germ layers *in vitro*. We have developed an efficient protocol for GABAergic neurons differentiation from pluripotent cells. We have developed fluorescent reporter system that provides both GABAergic neurons labeling in cell culture and FRET-based cell apoptosis detection. Established reporter iPSC lines and appropriate protocol of neural differentiation could be used for HD study, drug validation and screening.

Poster Board Number: F-3252

GENETIC MODULATION OF HUMAN IPSC DERIVED CHOLINERGIC NEURONS WITH MUTANT PRESENILIN 1 GENE GIVES RISE TO A POTENTIAL *IN VITRO* ALZHEIMER'S DISEASE MODEL FOR DRUG SCREENING.

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Alzheimer's disease (AD) is the most common cause of dementia characterized by impaired memory and cognitive dysfunction due to neurodegeneration.One of the many pathological features of the disease is the deposition of amyloid-beta-peptide (Aβ) as well as a loss of neuronal and synaptic cholinergic neurons in the cerebral cortex and hippocampus. This gives rise to the amyloid hypothesis that deposition of Aβ causes neurodegeneration. Furthermore, in familial AD, the likely cause of the disease is the mutation of the presenilin (PS) 1 gene coding of γ-secretase which is involved in processing Aβ from amyloid precursor protein (APP). The predicted

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growth of AD morbidity coupled with the socioeconomic impact of the disease places enormous pressure on the need to find an effect therapy. However, to date, the models of AD are challenging and a more humanized, scalable assay system is required to help better understand the disease and to aid identification of novel therapies. To achieve such an assay we utilized induced pluripotent stem (iPS) cells that have the ability to produce an endless quantity of human neurons, but more importantly, the cells can be genetically modulated to produce neurons displaying a diseased phenotype. Unlike using patient-derived cells, the disease model cells using gene transferred iPS cell technologies have some advantages to apply screening of drug candidate. Since the disease model cells have same genetic background of control expressing healthy phenotype, the effects of transgene can be clearly detected in the screening. Furthermore, the disease model cells are made from commercially available primary cells without recruiting the patients and associated some ethical procedures. To make AD model cells, we generated human iPS cells transferred mutant PS1 gene which is responsible for familial AD and differentiated into cholinergic neurons. The effect on production of Aβ40 and Aβ42 was measured using an AlphaLISA® Human Amyloid β1-40/1-42 immunoassay kit (Perkin Elmer) for high throughput screening (HTS). The results demonstrated an increased in the ratio of Aβ42/Aβ40 from cholinergic neurons carrying the mutant PS1 gene, compared to parental cell line. In conclusion, we have developed an *in vitro* AD assay, using iPS cell technologies that produces a specific disease phenotype which is similar to that seen in AD patients. Moreover, the phenotype can be quantified using immunoassay. Therefore we have developed a clinically relevant, potentially high throughput AD assay that is easily scalable due to the utilization of iPS cell technologies, which could play a significant role in finding the next generation treatments for AD.

Poster Board Number: F-3253

INDUCED PLURIPOTENT STEM CELLS FROM MELAS PATIENT SEGREGATES MITOCHONDRIAL HETEROPLASMY

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Mitochondrial disease is inherited through maternal transmission of mutated mitochondrial DNA (mtDNA) with disease phenotypes pre-set according to unpredictable mixture of mutant and wild-type mtDNA. Mitochondrial heteroplasmy is determined by stochastic segregation of mtDNA into discrete units during early embryogenesis as mtDNA replication is shut off during blastomere formation. Upon tissue-specific differentiation in the gastrulating embryo, mtDNA replication resumes and expands according to the pattern of mtDNA heteroplasmy to accommodate the oxidative requirements and maintain bioenergetic homeostasis. Since nuclear reprogramming resets a somatic cell into a glycolytic-dependent primitive pluripotent ground state, we hypothesized that bioengineering induced pluripotent stem (iPS) cells would trigger a reduction in mitochondrial dependency and initiate a process of mtDNA segregation at the cellular level to yield distinct metabotypes for individual iPS cell lines. Herein, fibroblasts originally obtained from a MELAS patient for clinical diagnostics demonstrated mitochondrial deficiencies with a reduced oxidative reserve compared to control fibroblast cell lines due to ~50% heteroplasmy in complex I at position 13513 in the ND5 subunit within a mosaic population. Nuclear reprogrammed patient-specific iPS cell lines demonstrated characteristic features of pluripotency coupled with multi-lineage differentiation. Individual iPS cell lines demonstrated metabolic conversion into glycolytic metabotype with reduced mitochondrial size and density compared to somatic cell types, mimicking pluripotent human cells with low levels of mtDNA and reduced oxidative reserve capacity. Comprehensive mtDNA sequence analysis of patient-derived iPS cells quantified the degree of heteroplasmy and confirmed MELAS-iPS cell lines that contained 50% heteroplasmy, along with iPS cells devoid of disease-causing mutations despite identical pattern of hypervariable regions within the remaining mtDNA. Furthermore, the degree of mitochondrial heteroplasmy was stable within each iPS cell line upon tissue-specific differentiation. Thus, nuclear reprogramming with reduced dependency on functional mitochondria produced isogenic pluripotent stem cell lines with or without disease-causing mitochondrial genotypes, revealing a novel platform to directly compare the natural spectrum of maternally encoded mitochondrial genotype/phenotype from pre-implantation biology to tissue-specific regeneration.

Poster Board Number: F-3254

INDUCED PLURIPOTENT STEM CELLS FROM CINCA SYNDROME PATIENTS AS A MODEL FOR DISSECTING SOMATIC MOSAICISM AND DRUG DISCOVERY

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Background: Derivation of induced pluripotent stem cells (iPSC) from fibroblasts enables us to study diseases under a new prospect. So far, disease modeling in neural or cardiac disorder has been reported, but there has been no report regarding immunologic disorder. Chronic Infantile Neurologic Cutaneous and Articular (CINCA) syndrome is a severe autoinflammatory disease caused by gain-of-function mutations of *NLRP3* gene. Mutant NLPR3 protein in macrophages causes overproduction of IL-1 beta, which leads to systemic inflammation. We previously identified patients of this syndrome with somatic mosaicism of *NLRP3* mutation. It has been controversial (1) whether only blood cells with NLRP3 mutation have abnormal function of cytokine secretion or all the cells in mosaic patients carry another unknown mutation and abnormal function and (2) whether the NLRP3-mutant macrophages with relatively small ratio actually cause the systemic inflammation. Results We obtained fibroblasts from two male patients of CINCA syndrome with somatic mosaicism. We obtained both wild-type and mutant clones from each patient's fibroblasts through transduction of OCT4, SOX2, KLF4 and cMYC with retroviral vector. iPSCs are differentiated into CD14+ macrophages via purification of CD34+KDR+ hematopoietic progenitors. Mutant iPSC-derived macrophages secreted higher amount of IL-1 beta when stimulated with lipopolysaccharides, while wild-type clones did not. Moreover, when mutant and wild type macrophages are mixed, they produced significantly more IL-1beta than only mutant cells did, suggesting the interaction of mutant and wild type macrophages in a patient's body. We extended the analysis of the interaction and obtained additional data. Furthermore, we examined the usefulness of iPSC-derived macrophages for drug screening.

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ALLELE-SPECIFIC KNOCKDOWN OF AN ALS ASSOCIATED TDP-43 MUTATION USING HUMAN INDUCED PLURIPOTENT STEM CELLS

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TDP-43 proteinopathies are a group of diseases with overlapping clinical-pathological features including: accumulation of phosphorylated, ubiquitinated and detergent insoluble TDP-43 in the cytoplasm of motor neurons; and C-terminal cleavage of TDP-43 producing fragments of 35 and 25 kDa. TDP-43 is an RNA/DNA binding protein that is encoded by the *TARDBP* gene and is mainly localized in the nucleus. TDP43 has been implicated in transcriptional repression, pre-mRNA splicing and translational regulation. Mutations in TDP-43 cause familial amyotrophic lateral sclerosis, and one of the most common mutations is the variant M337V. In order to investigate the potential use of allele-specific small interference RNA (siRNA) as a therapeutic, we screened five siRNAs that specifically target the TDP-43 ^{M337V} mutation. Initial screening was performed in HEK293 GFP-TDP-43 transfected cells and validated in induced pluripotent stem cells (iPSC) derived from an ALS patient's cells carrying M337V mutation. We identified one allele specific siRNA that reduces expression of HEK293 GFP-TDP-43 ^{M337V} transfected cells whereas GFP-TDP-43 ^{wt} remains unchanged. M337V neural stem cells (NSC) line derived from iPSCs showed two-fold higher TDP-43 in the cytosol compared to control ($P<0.001$). Following transfection with allele specific siRNA in NSCs ^{M337V} we observed a reduction of 72% in the cytosolic TDP-43 levels compared to cells transfected with scramble siRNA control ($P<0.001$). TDP-43 levels from NSC control line remain unchanged under siRNA allele specific transfection. We conclude that RNA interference can be used to selectively target the TDP-43 ^{M337V} allele in mammalian and patient's cells, thus demonstrating the potential for using RNA interference as a therapeutic tool for ALS.

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REPROGRAMMING NEUROBLASTOMA CELLS

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Background: Induced pluripotent stem (iPS) cells are reprogrammed embryonic-stem (ES) -like cells that carry the genomic information of its donor cell. Similar to ES cells, their differentiation should reflect cell differentiation in the developing embryo. We have hypothesised that iPS cells may be particularly suited to model childhood cancers, like neuroblastoma. Aim: To produce iPS cells from neuroblastoma cell lines. Methods: SK-N-SH cells were transduced with lentiviral vectors encoding Oct4, Sox2, Nanog, Lin28 and subsequently cultured in embryonic stem cell conditions. Potentially reprogrammed cells were selected by light microscopy looking for features of typical ES cell colonies. These were characterised for their expression of a well-established panel of pluripotency markers, karyotyped and differentiated by embryoid body formation. Results: We have succeeded in creating >26 iPS cell lines that maintain typical ES cell morphology; Of these, initially three cell lines were selected and characterised for pluripotency markers. They stained positive for surface markers SSEA3, SSEA4, and TRA-1-81 and expressed the endogenous transcription factors OCT4 and NANOG. When differentiated as embryoid bodies these cells up-regulated markers of the three germ layers (e.g. AFP, Brachyury, NCAM). The neuroblastoma karyotype of the original donor cell line was maintained. Conclusion and future: SK-N-SH cells can be re-programmed to produce neuroblastoma iPS cells. We are currently testing whether these reprogrammed cells are capable of forming neuroblastoma in immune-compromised mice. These novel cells may be a useful tool to explore the underlying mechanisms of neuroblastoma initiation, and to prioritise, or identify and evaluate, putative new treatment strategies.

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UNIQUE PRESERVATION OF NEURAL CELLS IN HUTCHINSON-GILFORD PROGERIA SYNDROME (HGPS) IS DUE TO THE NEURAL-SPECIFIC EXPRESSION OF THE MIR-9 MICRORNA

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One puzzling observation in patients affected with Hutchinson-Gilford progeria syndrome (HGPS), who exhibit a systemic and dramatic premature aging phenotype, is the absence of any conspicuous cognitive impairment. Recent studies based on induced pluripotent stem cells (iPSC) derived from HGPS patients cells have stressed the discrete lack of expression in control and HGPS neural derivatives of lamin A, a major isoform of *LMNA* initially produced as a precursor called prelamin A. Prelamin A defective maturation processing induces the production of toxic progerin in patients' cells nuclei. Here we show that miR-9, a neural specific microRNA, negatively controls lamin A and progerin expression in HGPS iPSC derived neural cells and is sufficient to attenuate nuclear blebbing in non-neural HGPS iPSC progenies. Altogether, our results support the hypothesis that the protection of HGPS patients neural cells from progerin accumulation relates to the physiologically restricted expression of the miR-9 microRNA to that specific cell lineage.

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GENERATION OF IPS CELLS FROM HUMAN T CELLS : THERAPEUTIC POTENTIAL OF IPS CELLS DERIVED FROM MATURE LYMPHOCYTES

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iPS cells can be induced from various types of somatic cells by reprogramming them using Yamanaka factors (Oct4, Sox2, Klf4, and Myc). They are reported to be very similar to ES cells in many respects, such as gene expression pattern, multipotency and differentiability . They are expected to be used as cell source for production of various types of cells to be used in regeneration medicine.

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In this study, we planned to use iPS cells as a progenitor source for immune cell therapy. For this purpose, iPS cells derived from a lymphocyte that shows a certain antigen-specificity are preferable, because antigen specificity is inherited to iPS cells made from the lymphocyte. For example, if iPS cell are produced from cytotoxic T cells specific to a tumor antigen, T cells generated from these T-iPS can be used in cell therapy for patients bearing cancer. We have succeeded in establishing iPS cells from human mature T cells (T-iPS cells), namely from whole CD3+ cells or CD4-CD8+ cells of cord blood as well as of adult peripheral blood. These T-iPS cells were confirmed to bear productively rearranged TCRβ chain gene. These T-iPS cells were differentiated into CD4+CD8+ double positive T cells and eventually into CD8+ single positive T cells expressing αβTCR in an *in vitro* co-culture system using OP9-DL1 stromal cells. Such T cell induction occurred more efficiently from T-iPS cells than from human ES cells or from iPS cells derived from other cell types such CD34+ cord blood cells or fibroblasts. To confirm that T-iPS cells can generate antigen specific T cells we established T-iPS cells from Mart1 (Melanoma antigen recognized by T cells 1) specific CTL cells. These Mart1 T-iPS cells were differentiated *in vitro* into CD4+CD8+ double positive T cells. Majority of these DP cells were found to be Mart-1 tetramer positive. We are now trying to induce mature T cells using NSG (NOD-SCID common γ-/-) mice. Because our Mart1 T-iPS cells are restricted to HLA-A2, in this experiment setting we used NSG mice carrying HLA-A2.

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TRANSIENT EXPRESSION OF REPROGRAMMING FACTORS *IN VIVO* LEADS TO WILMS'-LIKE TUMOR DEVELOPMENT IN THE KIDNEY

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Induction of 4 transcription factors, Oct3/4, Klf4, Sox2, Myc in differentiated somatic cells has yielded induced pluripotent stem cells (iPSCs). Although iPSC generation does not require changes in genomic sequence, iPSCs acquire indefinite growth potential; a key characteristic shared with cancer cells. In this study, we generated a reprogrammable mouse in which reprogramming factor expression can be controlled temporally *in vivo* by treatment with doxycycline (Dox), and examined the effects of factor expression in somatic cells and tissues. Induction of reprogramming factors *in vivo* caused active cell proliferation and a rapid expansion of dysplastic lesions in epithelial tissues. Prolonged expression of reprogramming factors caused the invasive growth of dysplastic cells, leading to cancer development in various organs, including the intestine and skin. Expansion of dysplastic cells was observed in the pancreas and kidney where cells do not divide actively under normal physiological conditions. Interestingly, such dysplastic cells disappeared shortly after the withdrawal of Dox treatment, possibly reflecting an epigenetic memory for cellular identity. Following longer periods of reprogramming factor induction, we observed dysplastic proliferating cells in the kidney, which continued neoplastic growth even after Dox withdrawal. These Dox-independent tumor cells grew invasively into the adipose tissue surrounding the kidney. Histological analysis revealed that the Dox-independent kidney tumor resembles Wilms' tumor, a common pediatric kidney cancer. In addition, microarray analysis demonstrated that many upregulated genes in the Dox-independent kidney tumors were similarly upregulated in Wilms' tumors. Our results suggest that aspects of cellular reprogramming may be involved in the development of pediatric cancers, and our reprogrammable mouse system could present a suitable model system to study these processes.

Poster Board Number: F-3260

USE OF INDUCED PLURIPOTENT STEM CELLS TO MODEL SELECTIVE NEURONAL FAILURE IN NIEMANN PICK TYPE C1

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Niemann-Pick type C1 (NPC1) is a progressive and uniformly fatal inherited pediatric dementia, characterized by defects in intracellular cholesterol trafficking. NPC1 is caused by loss of function of the endosomal-lysosomal membrane transporter NPC1. Disruptions in this transport system result in the accumulation of cholesterol and glycolipids in the lysosomal compartment, triggering progressive cerebellar, hippocampal, and cortical neurodegeneration. NPC1 has intriguing similarities with Alzheimer's disease, suggesting a common pathogenic mechanism. Previous research in our lab utilizing genetically engineered NPC1 knockdown (KD) human embryonic stem cells (hESC) suggests that disrupted turnover of mitochondria by autophagy may be a major factor causing selective neuronal failure. Utilizing retroviral reprogramming methods we have generated and characterized a set of induced pluripotent stem cells (IPSC) from NPC1 null and severe hypomorph patient fibroblasts. We efficiently induce neuronal differentiation of control and NPC1 hIPSCs using specific growth factor combinations and enrichment of neuronal populations by fluorescence activated cell sorting (FACS). Our main goal is to use patient specific NPC1 neurons, and hESC-derived NPC1 knockdown neurons previously generated in our lab to probe alternative mechanisms of disease that will lead to effective therapeutic approaches for NPC1 and related disorders. Specifically, we are testing the hypothesis that NPC1 mimics a state of cholesterol starvation, inducing excessive activation of autophagy as an alternative route to release cholesterol from the lysosome, and that imbalanced autophagy is a major contributor to selective neuronal failure, triggering downstream pathologies typical of disease progression including synaptic defects. The use of hIPSCs to study human neurodegenerative disease is in the early stages of development and validation of the use of these cells and their derivatives to model human disease is crucial. Parallel analysis of neurons derived from NPC1 KD hESCs and patient specific hIPSCs is a powerful approach that will begin to assess the role of genetic heterogeneity in generating neuronal phenotypes, and therefore will help cross validate the use of hIPSCs for the study of neurodegenerative diseases.

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IDENTIFICATION OF NOVEL BIOMARKERS FOR VASCULAR COMPLICATIONS ASSOCIATED WITH AUTOSOMAL DOMINANT POLYCYSTIC KIDNEY DISEASE USING PATIENT-SPECIFIC IPSCS

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Autosomal dominant polycystic kidney disease (ADPKD) is the most prevalent, potentially lethal, monogenic disorder, characterized

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by the development of multiple renal cysts and various extrarenal manifestations. Cardiovascular complications are the main cause of death in ADPKD patients and intracranial aneurysms, causing subarachnoid hemorrhage, are among the most serious. The pathogenesis of vascular lesions as well as cyst formation remains largely unknown. Here we report the derivation of induced pluripotent stem cells (iPSCs) from skin fibroblasts from seven ADPKD patients, among whom four had intracranial aneurysms. These iPSCs differentiate into vascular endothelia and smooth muscle cells *in vitro*, which recapitulate the defective intracellular Ca2+ regulation, similar findings to those reported in vascular cells of mouse ADPKD models. Furthermore, by microarray analyses, we have identified several molecules whose expression levels are specifically altered in the iPSC-derived vascular cells from ADPKD patients and in those from ADPKD patients with aneurysms. Among the molecules, both mRNA and protein expression of an enzyme and its secretion into culture media show statistically significant elevations in the iPSC-derived endothelia from ADPKD patients with aneurysms as compared to the patients without. These results suggest that vascular cells differentiated from patient-specific iPSCs can be used for studying the mechanisms of vascular complications in ADPKD and for identifying novel molecular diagnostic and therapeutic targets in ADPKD.

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GENERATION OF MUTANT HUMAN INDUCED PLURIPOTENT STEM CELLS AS A CELLULAR MODEL FOR SPINOCEREBELLAR ATAXIA TYPE 2

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Introduction: Spinocerebellar ataxia type 2 (SCA 2) is caused by triple nucleotide repeat (CAG) expansion in the coding region of the ATAXN2 gene on chromosome 12, which produces an elongated, toxic polyglutamine tract, leading to Purkinje cell loss. There is currently no effective therapy. One of the main obstacles that hampers therapeutic development is lack of an ideal disease model. In this study, we have generated an affected human SCA 2 iPS cell line and a wild type iPS cell line. We further compared their neural differentiation and characterized SCA 2 iPS cells as an *in vitro* cell model. Materials and Methods: The study was approved by the UF institutional Review Board. The SCA 2 subject was a 30 year old male with 20/44 CAG repeats. Normal control was a non-affected 51 year old male. Skin samples were taken by punch biopsy. Dermal skin fibroblast cells were generated by primary culture of skin explants. Passage 3 of skin fibroblasts were used for reprogramming. Traditional Yamanaka factors (hOct4, hSox2, hKlf4, hc-Myc) were transduced by retroviral infection. The iPS cell lines were characterized by morphology, Karyotyping, RT-PCR and immunofluroscence assay of stem cell markers. Pluripotency was assessed by *in vitro* Embryoid Body-mediated differentiation. The generation of neural stem cells(NSC) and *in vitro* neural differentiation were assessed. NSC and neural cells differentiation were monitored by morphology and immunofluroscence stains of specific NSC marker (nestin), neuronal markers (neurofilament H, β-tubulin III) and astrocytic marker (GFAP). Pathognomic marker of SCA 2 was detected by a immunocytochemical staining of polyglutamine inclusion bodies. Time-lapsed neuronal growth pattern was compared between SCA 2 and normal cells. The stability of the expanded CAG repeats of SCA 2 during reprogramming and neural differentiation were also assessed. Results: SCA 2 iPS and normal iPS clones show typical stem cell growth pattern in culture with high nuclear/cytoplasm ratio with normal karyotype. The iPS colonies maintain the same growth pattern through subsequent passages. All iPS cell lines express stem cell markers by RT-PCR and immunocytofluorescence (Oct4, Nanog, Sox2, SSEA4) and differentiated into three embryonic germ layer cells *in vitro* using Embryoid Body formation. Upon *in vitro* neural differentiation, normal iPS cells underwent normal differentiation steps from neurospheres to neural rosettes, neural stem cells and neural cells. However, SCA 2 iPS cells showed abnormality in neural rosette formation but successfully differentiated into neural stem cells and subsequent neural cells. SCA 2 neural cells contain pathognomic marker of polyglutamine inclusion bodies. Time-lapsed neural growth assay indicate terminally differentiated SCA 2 neural cells are short-lived compared to normal neural cells. The expanded CAG repeats of SCA 2 were stable throughout reprogramming and neural differentiation. Conclusions: We have established the first disease-specific human SCA2 iPS cell line. The mutant iPS cells have the potential for neural differentiation. The neural stem cells, neurons and astrocytes harboring mutations are ideal for the study of SCA 2 pathogenesis. These cells can also function as a translational platform for therapeutic drug development and cell-based therapy of polyglutamine disorders.

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IPS-DERIVED KIDNEY PODOCYTES FROM PATIENTS WITH GENETIC KIDNEY DISEASE: APPLICATIONS FOR DISEASE MODELING AND THERAPEUTIC SCREENING

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The increasing incidence of end-stage renal disease (ESRD) is a worldwide health crisis responsible for considerable burden of illness and premature mortality. Alport Syndrome and polycystic kidney disease (PKD) are inherited genetic disorders that lead to a progressive loss of kidney cells resulting in declining organ function and ESRD. The reprogramming of somatic cells to induced pluripotent stem (iPS) cells has attracted considerable attention for disease modeling, drug screening and regenerative medicine and we have recently shown that iPS cells can be derived from human kidney cells (J Am Soc Nephrol, 2011). In the present study, fibroblasts and kidney cells grown from skin and kidney biopsies of patients with Alport Syndrome and polycystic kidney disease (PKD) were reprogrammed by retroviral transduction (OCT4, SOX2, KLF4, and c-Myc) and subsequently differentiated towards kidney glomerular podocytes. The DNA methylation profiles of skin-derived iPS cells were analyzed by bisulfite sequencing using the OCT4 promoter, compared to kidney cell-derived iPS cells. The iPS colonies were analyzed for stem cell marker expression using immunofluorescence microscopy and qPCR. The pluripotent capacity of the undifferentiated iPS cells was tested by the formation of teratomas following injection into immunodeficient mice and the differentiation into embryoid bodies. Further to this, we have developed a reliable protocol that efficiently directs the differentiation of iPS cells towards kidney glomerular podocytes. The disease-specific iPS cells resembled human embryonic stem cells (hES) in morphology and gene expression localizing for OCT3/4, SSEA-4, TRA-1-60 and TRA-1-81 proteins. Using qPCR, the iPS cells expressed stem cell marker genes and exhibited silencing of the retroviral transgenes by passage four. DNA methylation profiles showed that fibroblast

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and mesangial-derived iPS cells had OCT4 methylation patterns similar to hES cells, but different to the primary target cells. iPS cells formed embryoid bodies and expressed markers of all three germ layers by immunostaining and RT-PCR. The injection of undifferentiated iPS colonies into immunodeficient mice formed teratomas, thereby demonstrating pluripotency. Upon embroid body differentiation towards the mesoderm lineage and kidney progenitors the iPS-derived podocytes shared a similar morphological phenotype and expressed proteins and mRNA comparable to primary human podocytes. The present study established iPS cell lines and kidney podocyte progenitors from the somatic cells of Alport and PKD patients that maintain the disease genotype and phenotype, advancing the potential of human iPS-derived kidney cells for modeling the genetic disorder and for the screening of new drug compounds. This will offer alternatives to the limited framework of existing clinical options.

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DEVELOPING IPS CELL TECHNOLOGY FOR TREATING HIV INFECTION

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The discovery of iPS cell holds great promise in regenerative medicine since it is possible to produce patient specific pluripotent stem cells from affected individuals for autologous treatment. Our study is aimed towards treatment of HIV infection with autologous hematopoietic stem cell (HSC) transplantation through iPS cell therapy. It was reported more than 15 years ago that individuals homozygous for the Δ32 mutation of the chemokine receptor, CCR5 are highly resistant to HIV infection. Recently, after an HIV-infected patient with acute leukemia received stem cell transplants from a HLA compatible donor homozygous for this mutation, he has not required antiviral medications and remains free of detectable viremia . Hence, a possible approach to therapy is to prepare iPS cells from infected patients, knock out both wild-type CCR5 alleles or replace them with the Δ32 mutation and differentiate them into HSC for auto-transplantation. In this study we propose to demonstrate the feasibility of this approach by rendering cells obtained from normal individuals resistant to HIV infection. In order to minimize genomic DNA damage, we avoid techniques that involve integrating viral vectors or introduce double-strand DNA breaks. Using the non-integrating cytoplasmic Sendai viral vectors (CytoTuneTM iPS), we could generate iPS cells efficiently (0.06-0.25%) from adult CD34+ and peripheral blood mononuclear cells (PB-MNC). After 3-5 passages, Sendai viral genome could not be detected by real-time quantitative RT-PCR or immunostaining for Sendai viral proteins. To exclude T cell or B cell derived iPS clones reprogrammed from PB-MNC, clonality assays was performed and showed that only 1 out of 14 lines tested was derived from B lymphocyte. To knock out the CCR5 gene or replace it with the CCR5Δ32 mutation by homologous recombination, we used BAC based vectors and obtained frequencies of mutation of 4-6%. Mutation of the 2nd allele is in progress. Using the spin EB method, we showed that these blood cell derived iPS cells could efficiently be differentiated into HSC without the need of co-culture with either mouse or human stromal cells. We obtained ~50% CD34+ and ~25% CD34+/CD43+ HSC that could be differentiated into mature blood cells including monocytes and lymphocytes. These blood cells prepared from the mutated iPS cells will be tested for their resistance to HIV in vitro. The CD34+ cells from them will be transplanted into immunodeficient mice and resistance to HIV infection tested *in vivo*. Our results so far demonstrate that the Sendai viral vectors are very efficient in reprogramming adult CD34+ and PB-MNC and the iPS cells so generated are vector and reprogramming factor free. Their mutation and differentiation into HSC can provide an approach for curing HIV infection.

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EXPLORING NEURONAL PHENOTYPES ASSOCIATED WITH PSYCHIATRIC DISEASE IN PATIENTS WITH 22Q11 DELETION SYNDROME

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Neuropsychiatric disorders such as autism and schizophrenia are challenging targets for therapeutic drug development because of the difficulty of identifying novel molecular targets in humans. The advent of induced pluripotent stem cells (iPSCs) provides an opportunity to generate neurons from patients with psychiatric disease that allows the subsequent identification of signaling defects in these cells. The deletion of the 22q11 chromosomal region gives rise to a multisystem disoder, which has been consistently associated with both schizophrenia and autism. We have generated iPSCs from a cohort of patients with 22q11DS that have been diagnosed with schizophrenia or autism. Using single cell gene expression, RNAseq, calcium imaging and neurite morphology we identified a series of interesting phenotypes in patient-derived neurons when compared to neurons derived from healthy controls. These results provide biological insights into the underlying cellular defects that lead to psychiatric disorders.

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ESTABLISHING A PATIENT-SPECIFIC IPSC MODEL FOR HIRSCHSPRUNG'S DISEASE AND OTHER NEURAL CREST-ASSOCIATED DISEASES

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Hirschsprung's disease (HSCR) is a congenital disease which is caused by the partial colonisation of enteric neural crest (NC) cells along the bowel where these cells fail to proliferate, differentiate or migrate during fetal development. The absence of ganglia especially in the hindgut region results in bowel obstruction. In order to study the disease etiology of HSCR, we employed the iPSC technology for establishing the human model for HSCR. We have selected a patient carrying a risk allele (T) in rs2435362 of RET gene who exhibits partial agangliosis accompanied by atrial and ventricular septal defects (ASD/VSD). It is speculated that defects in both the enteric and cardiac NC cells causes a reduction in ganglia and smooth muscle tissues leading to congenital structural malformation which are relatively common in other HSCR patients. We reprogrammed the patient's fibroblast cells into iPS cells by ectopic expression of four reprogramming factors. Three patient-specific iPS cell lines were currently obtained. They were ES-like, expressing the pluripotency markers and with low DNA methylation levels of CpG sites in the promoter regions of NANOG and OCT3/4. Importantly, they could generate teratoma comprising all three germ lay-

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ers when they were injected in SCID mice, further corroborating the cells had acquired pluripotency. Subsequent differentiation experiments revealed that these HSCR iPS cells were able to differentiate into NC cells of a comparable capacity as that of the control iPS cells (IMR90). In addition, these iPS-derived NC cells were multipotent and could commit to both neurogenic and smooth muscle lineages under defined differentiation conditions. Nevertheless, in general, all the HSCR-iPS cells showed a lower competency to form neurons and smooth muscle cells, suggesting that differentiation defects of NC may represent a cause of HSCR and other NC-associated disorders. Taken together, these results substantiate the potential use of our patient-specific model to study the etiology of HSCR and other NC-associated diseases.

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HUMAN IPSC-DERIVED MESENCHYMAL STEM CELLS MODULATE MYOCARDIAL REGENERATION THROUGH REDUCING OXIDATIVE STRESS AND RESTORING CARDIAC PROGENITOR CELLS IN ANTHRACYCLINE CARDIOMYOPATHY

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Background: Anthracycline is one of the most effective anticancer treatments ever developed for various malignancies. However, its clinical implementation is markedly hampered by its severe cardiotoxicity. Depletion of cardiac progenitor cells (CPCs) by anthracycline treatment has been proposed as a novel concept in the development of anthyclincine-induced cardiomyopathy. This study aims to study therapeutic efficacy and mechanisms of human induced pluripotent stem cell-derived mesenchymal stem cells (iPSC-MSCs) against doxorubicin induced-cardiomyopathy. Methods and Results: Administration of doxorubicin (DOX) in mice resulted in a dilated myopathy, heart failure and death. To test therapeutic efficacy of iPSC-MSC in attenuation of anthracycline-increased induced oxidative stress depletion of CPC pool and myocardium apoptosis, mice were randomly assigned to receive phosphate-buffered saline (Dox group); 3.0x105 human BM-derived MSCs (BM-MSCs group) or 3.0x105 iPS-derived MSCs (iPS-MSCs group) injections intramyocardially. Among all groups at 3-weeks post-transplantation, iPSC-MSC treatment displayed the most prominent efficacy in restorationof heart dysfunction and CPCs depletion, decrease of oxidative stress and myocardium apoptosis, and promotion of cardiomyocyte recycling. Compared to adult BM-MSCs, a differential paracrine capacity, and a greater cellular retention and differentiation potential with lower level of MHC expression in iPSC-MSCs were observed that may attribute to a higher efficacy of iPSC-MSC against Doxorubicin cardiomyopathy. Conclusion: our results indicate human iPSC-MSC exhibits a greater protective efficacy than BM-MSC against anthracylcine-induced cardiomyopathy and raise the possibility that iPSC-MSCs can be administrated to individuals who are particularly sensitive to the cardiotoxicity of anthracyclines for prevention and/or management of heart failure in future.

Poster Board Number: F-3268

REPROGRAMMING OF WRINKLY SKIN SYNDROME PATIENT FIBROBLASTS INTO INDUCED PLURIPOTENT STEM CELLS

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Autosomal recessive cutis laxa type II (also called Wrinkly Skin Syndrome, WSS) describes a group of syndromal disorders that often associated with a progeriod appearance, lax and wrinkled skin, osteopenia and mental retardation. Recent work has revealed that mutations in PYCR1 (pyrroline-5-carboxylate-reductase 1) gene are the cause of the disease. PYCR1 is an enzyme involved in proline metabolism and localizes to the mitochondrial matrix. It catalyzes the obligatory and final step of *de novo* proline synthesis. However, the levels of proline in WSS patient serum or cultured fibroblast cells are within normal range. Studies in Xenopus and zebrafish have shown that knock down of PYCR1 transcripts in embryos lead to stunted growth, obvious skin defects with disorganized skin epithelium and increased rate of apoptosis. This suggests that loss of PYCR1 may leads to developmental defects through increased apoptosis. Studies of PYCR1 function in human tissues are not available. Unlike Xenopus which has one PYCR gene and no paralogs, human PYCR1 has two paralogs, the highly similar PYCR2 and more distantly related PYCRL. In WSS patient fibroblasts, the expression level of PYCR2 and PYCRL was found normal. In order to study the role of PYCR1 in the pathogenesis of WSS, we plan to generate WSS- iPS cells as a model system. In this study, fibroblast lines from two patients with same PYCR1 mutation (K215-D319 del) and two normal lines were reprogrammed into iPSCs. WSS-iPSC clones were found with no expression of PYCR1 protein, similar to that of patient fibroblasts. All clones expressed high level of endogenous pluripotent markers, similar to human ESC lines. There is no growth retardation or apoptosis observed in WSS-iPSCs. WSS-iPSCs can give rise to three germ layers of tissues in teratoma formation assay. We further confirmed that PYCR2 and PYCRL expression in iPSCs kept stable and were not compensatory up-regulated. These results suggest that PYCR1 is dispensable in iPSC generation and maintenance. WSS-iPSC may serve as a reliable model system to study the mechanism of PYCR1 in tissue development, esp. neural, skin and bone formation. The results of accessing mitochondria functions in patient iPSCs will also be shown.

Poster Board Number: F-3269

PROTEIN BASED HUMAN INDUCED PLURIPOTENT STEM CELLS AS A POTENTIAL DONOR FOR PARKINSON'S DISEASE CELL THERAPY

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Human embryonic stem (hES) cells, derived from preimplanted embryonic blastocyst, are regarded as a desirable cell source for cell replacement therapies. In addition to safety and ethical concerns, however, clinical level of cell transplantation may require donor cells of which MHC genes are compatible to those of the recipient. In an effort to generate histocompatible stem cells, several groups have established ES cell-like pluripotent stem cells derived from human adult tissue cells by reprogramming (human induced pluripotent stem cells; hiPS cells). To test a possibility of hiPS cells in therapeutic use for Parkinson's disease (PD) characterized by degeneration of dopamine (DA) neurons in the midbrain substantia

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nigra, this study aims at derivation of midbrain-type DA (mDA) neurons from hiPS cells and evaluation of the hiPS-derived DA neuron properties, functions, and safety in clinical use. Co-culture protocol with the stromal cell-line MS5 was applied on the differentiation of the hiPS cells which has been established from foreskin fibroblasts by lentiviral exogene delivery of Oct3/4, Sox2, Nanog, LIN28. Similar to hES cell differentiation, hiPS cells efficiently differentiated toward midbrain-type neural precursor (NP; hiPS-NP) cells, which in turn yielded an enriched population of mDA cells exhibiting the expressions of the markers related to the DA neuron, tyrosine hydroxylase (TH) and dopamine transporter (DAT), midbrain-specific markers engrailed-1 (En1), Nurr1, A9 midbrain DA Neuron-specific marker G-protein regulated inward-rectifier potassium channel subunit2 (Girk2). Upon transplantation, the hiPS-NP cells survived for 4 weeks of post-transplantation period, differentiated into mDA neurons which were integrated to host striatum and induced significant behavioral restorations in 2 out of 4 PD rats. Next, we compared hiPS-NP (DA) cell properties with those of NP cells derived from hES cells (hES-NP). Previous studies have shown that the proliferative and DA neurogenic properties of hES-NP cells are unaltered after long-period of NP cells expansion. By contrast, proliferation of hiPS-NP cells was sustained only for short-term period: three times after NP passages, proliferation of hiPS-NP cells were not induced with a decrease of telomerase activity. In contrast to none or slight expressions of undifferentiated markers in hES-NP cells, relatively abundant expression of the undifferentiated markers and cells expressing Oct3/4 were detected in the cultures for hiPS-NP (DA) cells. RT-PCR analyses using the primers specifically detecting the undifferentiated genes exogenously introduced showed that expressions of the exogenes were not abolished but continued after differentiation. Furthermore, the expression of exogenes were modulated by several intracellular signalings including that mediated by cAMP. Taken together, this study indicates a promising prospective of hiPS-NP cells as a source for PD therapy, but problems in cell maintenance *in vitro* and safety in clinical use are remained to be further solved.

Poster Board Number: F-3270

ION CHANNELS PROPERTIES OF EPILEPTIC NEURONS DIFFERENTIATED FROM HUMAN-INDUCED PLURIPOTENT STEM CELLS

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Dravet syndrome (DS) is a kind of severe myoclonic epilepsy of infancy (SMEI), which is found closely associated with the mutation of voltage-gated sodium channels (Nav1.1), SCN1A gene. Nav1.1 mainly expresses in the central and peripheral nervous system. It plays a crucial role in the initiation and propagation of action potential and is an important regulator for neuronal excitability. There have been studies examining the functional effect of SCN1A mutations *in vitro* expression system. Researches exploring the linkage between SCN1A mutations and the genetic disease in Nav1.1 knockout mice have also been carried out. As the majority of studies to date rely on exogenous over-expression of SCN1A mutant in HEK cells and transgenic mice, the impact of endogenous SCN1A mutations on functional human patient neurons remains unclear. The purpose of our research is to establish disease cell model using DS patient iPS cell-derived neurons. Such neurons will then be used to study what functional change may result from the Nav1.1 mutation and how the exact functional abnormality will further cause DS. In this research, skin fibroblasts of DS patient and normal human (control) were reprogrammed and directed to differentiate into neurons. Immunoassaying and patch clamp technique were performed for neurons identification. The normal human iPS-derived neural cells have been well characterized by electrophysiological recording. Also, electrophysiological assay showed functional active neurons from DS patient derived iPS cells were generated. These neurons displayed single action potential, TTX sensitive sodium current and TEA-Cl sensitive potassium current after certain stimulatory signal was given. Our study demonstrated that iPSCs can generate electrically active neurons with characteristics of their natural counterparts. Functionally analyzing these cells may help us to understand the feasibility of using iPSCs-derived corrected neurons in epileptic therapy and *in vitro* cell model for drug screening.

Poster Board Number: F-3271

HUMAN INDUCED PLURIPOTENT STEM CELL MODEL OF BARTH SYNDROME

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Barth Syndrome (BTHS) is an X-linked recessive mitochondrial disorder caused by loss-of-function mutations in the Tafazzin (TAZ) gene. TAZ encodes an acyltransferase involved in the metabolism of cardiolipin (CL), a unique phospholipid nearly exclusively localized to the mitochondrial inner membrane. Functional deletion of TAZ impairs proper maturation of CL, leading to mitochondrial dysfunction and various phenotypes including heart failure, myopathy, neutropenia, and growth retardation. Interestingly, patients experience variability in symptoms, age of onset, and disease progression. Among the clinical features of the disease, heart failure is most critical for early lethality and investigation using heart tissue is most relevant. Unfortunately, access to heart tissue is extremely limited and current animal BTHS models are not sufficient to investigate this direction. For these reasons, we generated induced pluripotent stem cells (iPSCs) and *in vitro* differentiated cardiomyocytes to develop a useful and highly relevant human model for BTHS. BTHS iPSCs were successfully generated through conventional retroviral transduction of Oct4, Sox2, c-Myc, and Klf4. They demonstrated apparently normal morphology and growth rate, normal karyotype, expression of pluripotency specific markers including SSEA4, Nanog and Oct4, and the ability for teratoma formation in immunocompromised mice. When we examined mitochondrial phospholipid status using MALDI-TOF, BTHS cells showed a marked reduction in mature CL recapitulating the disease phenotype *in vitro*. Differentiation to cardiomyocytes using high density monolayer and addition of Activin A and BMP4 growth factors allowed us to examine mitochondrial morphology and dysfunction in the target tissue. BTHS iPSCs provide a valuable model to study mechanisms underlying how TAZ mutation and other unknown genetic factors in BTHS patients lead to cardiac abnormality. Further, these cells will become useful to develop novel therapeutic interventions.

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DIFFERENCES IN APOPTOTIC RESPONSE TO DNA DAMAGE OF PLURIPOTENT STEM CELLS FACILITATES PHARMACOLOGIC PURGING OF TERATOMA-FORMING STEM CELLS

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Pluripotent stem cells offer an unlimited potential for regenerative medicine, yet harnessing the therapeutic capacity while minimizing the risk of uncontrolled tumor formation remains a formidable challenge. As partially differentiated progenitor cells derived from pluripotent starting sources have been the focus of bioengineering efforts designed to generate therapeutic products, the uncertain risk of residual pluripotent stem cells requires a targeted approach to exclude contaminating cell types. In this study we aimed to define a toxicity strategy that could selectively purge pluripotent stem cells from partially differentiated progenitor cells to avoid the risk of uncontrolled cell growth in a mixed population, thereby increasing the safety of a therapeutic cell population. In response to increasing doses of genotoxic agents, annexin-V binding assays demonstrated that mouse embryonic stem cells and induced pluripotent stem (iPS) cells, in contrast to somatic cell types, are hypersensitive to apoptotic induction in response to DNA damage. Notably, this hypersensitivity of pluripotent stem cells is abrogated upon *in vitro* differentiation, with the IC50 increasing nearly 2-orders of magnitude. Quantitative RT-PCR and western blotting demonstrated that this response is mediated through upregulation of the BH3-only protein Puma. Furthermore, mouse teratoma studies and transcriptional analysis demonstrated that etoposide treatment of partially differentiated progenitors derived from pluripotent stem cells could be purged of residual stem cells rendering the progenitor population of cells refractory to teratoma formation. Collectively this study establishes that the phenotype of a hypersensitive apoptotic response to DNA damage of mouse pluripotent stem cells can be exploited to decrease dysfunctional tumor formation and increase the safety of a therapeutic cell population without requiring long-term differentiation prior to transplantation.

Poster Board Number: F-3273

A HIGH CONTENT DRUG SCREENING PLATFORM FOR HUMAN MOTOR NEURONS FROM NON-INTEGRATING IPS CELLS OF MULTIPLE SPINAL MUSCULAR ATROPHY PATIENTS

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Background: Spinal muscular atrophy (SMA) is a recessively inherited pediatric neuromuscular disease characterized by degeneration of spinal motor neurons (MNs), often resulting in death. Survival motor neuron 1 (SMN1) deficiency is the known genetic component. SMN protein, the ubiquitously expressed SMN1/2 gene product, specifically affects MNs in SMA via unknown mechanisms. Increases in SMN (whole cell and punctate nuclear gem structures) expression remain one of the best predictors of drug success in SMA, but these assays have been performed in patient fibroblasts or tumor lines, thus necessitating analysis of MNs that are adversely sensitive to diminished SMN. A new paradigm in drug discovery is the generation of induced Pluripotent Stem Cells (iPSCs) from SMA patients, which can then be differentiated to study pathophysiologically affected MNs. Purpose: To develop a consistent protocol for differentiating MNs from multiple SMA patient iPSC lines amenable for High Content Imaging (HCI) and perform large-scale chemical screens targeting SMN up-regulation and anti-apoptotic pathways. Methods: Patient fibroblasts were reprogrammed to iPSCs under 5% O2 using non-integrating plasmid-based factors: OCT3/4, SOX2, KLF4, L-MYC, LIN28, p53 shRNA, and small molecules on feeder-free Matrigel in mTeSR1. Pluripotency was confirmed with standard methods. SMN levels were analyzed by whole-cell lysate immunoblotting. Spin embryoid-body formation method and directed differentiation in neural induction media supplemented with retinoic-acid, purmorphamine, and neurotrophins yielded SMA iPSC-MNs plated on "motoneuron Matrigel" substrata in 384-well plates. MNs were treated for 10 days with Screen-well REDOX library (Enzo) prior to fixation. SMN (gem and cytoplasmic) identification was performed using an automated algorithm developed in-house on Image Express Micro (IxM) MetaExpress system. Results: Non-integrating iPSC lines were successfully generated from five healthy subjects and four SMA patients. SMA iPSCs maintained disease genotype and manifested phenotype showing selective degeneration in MN cultures over time, measured by increased apoptosis, activation of caspases-8, and -3, and increased Fas ligand levels. Blocking this apoptotic pathway mitigated SMA iPSC-MN loss. Select compounds identified by pre-screening in other SMA models were tested for SMN up-regulation in SMA iPSC-MNs. Only few compounds tested positive while most tested negative. Further, iPSC-MN differentiation optimized for HCI (384-well plates) yielded MN progenitor populations (>20%) at distinct stages of differentiation that cryopreserved with excellent post-thaw cell viability (>90%). After REDOX library treatment of four SMA patient iPSC-MNs, SMN up-regulation was identified specifically in MNs and utilized as the primary screening assay. Co-localization of SMN gems was detected in MN nuclei labeled for progenitors (NKX6.1+, ISLET1+, HB9+) and mature neuronal cells (MAP2a/b+, CHAT+) using the automated algorithm. Preliminary chemical "hits" are being validated further. Conclusions: We have developed a reliable stem cell model of SMA amenable to high-content screening in patient-derived MNs. Chemically diverse libraries will be screened for raising SMN protein to non-diseased levels. "Hits" will then be tested in phenotype-based secondary screens that block apoptosis and rescue MN loss, potentially leading to a cogent drug development strategy for SMA.

Poster Board Number: F-3274

THE ACTIVATION OF C-MYC AND LIN28B CAUSES IPSC TUMOR FORMATION DURING *IN VITRO* CARTILAGE DIFFERENTIATION

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Induced pluripotent stem cells (iPSCs) show great potential for regenerative medicine applications; however particular lines can acquire pro-oncogenic potential, which can be revealed after transplantation. We have observed that certain iPSC lines form abnormal adenocarcinoma-like tumors following three dimensional (3D) cartilage tissue engineering. The *in vitro* cartilage tissue engineering approach may be an excellent system for recognizing a pro-oncogenic state in select human iPSC lines without having to transplant into animal, as well as for exploring the key factors in tumor formation as we could identify any abnormal differentiation by histological analysis. In this study, we focused on the key regulators causing this pro-oncogenic state in these iPSC clones. Following 6 weeks of culture using micro-mass approach which we

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have modified previously, both embryonic stem cells (ESCs) and iPSCs formed the hallmarks of cartilage *in vitro*. However, certain iPSC clones showed the ectopic adenocarcinoma-like tumor formation within cartilage template as the abnormal differentiation. When we compared the expression profiles of several pro-oncogenic factors in ESCs and pro-oncogenic iPSCs during cartilage differentiation, we observed significant differences in the expression profile of the proto-oncogenes endogenous c-MYC and LIN28B. Both c-MYC and LIN28B were also expressed in only abnormal tumor. The siRNAs directed against c-MYC or LIN28B significantly reduced tumor formation during cartilage differentiation. These results indicated that the activation of c-MYC and LIN28B caused tumor formation in pro-oncogenic iPSC clones. Evaluating safety is the foremost issue in human iPSCs for clinical application. The understanding the key regulators toward tumor formation would help to evaluate the safety of human iPSC lines. The data present here indicates that c-MYC and LIN28B are part of an epigenetic switch mechanism promoting tumor formation in pro-oncogenic iPSC clone. It is difficlut to predict oncogenic nature of iPSCs in undifferentiation states. The monitoring of c-MYC and LIN28B activation during differentiation would help to identy pro-oncogenic iPSC clones.

Poster Board Number: F-3275

BASIC RESEARCH OF THE IMMUNO ASSAY BY THE CAPILLARY ISOELECTRIC FOCUSING FOR EFFICIENT ACQUISITION OF THE IPS CELL

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The practical application the induced pluripotent stem cell (iPSC) is expected to be in the field of regenerative medicine. Various methods to create iPSCs such as combination with siRNA, and drugs, have been developed, however there are many problems to be solved developing the clinical application. Among the one problem is to evaluate of them characters in iPSCs. Because there are individual specificities in the established iPSCs, the brief evaluation of their characters by molecular analyses is needed. Standardization of iPSCs needs skillful techniques and continuous culture to maintain the cells, those spend a large labor. Thus a simple evaluation method to select the founder colony of iPSCs that should have a suitable ability is needed for improvement of research. The aim of this study was to establish a simple and easy method to analyze proteins, such as the stem cell markers that could help the easy screening of characteristics of iPSCs. Especially, the capillary isoelectric focusing (cIEF) that could separate and detect proteins selectively was chosen, and the basic experiment has been achieved for establishment of this method. We demonstrated that the SOX2 protein, one of the stem cell markers, could be detected by this new cIEF method to identify the immuno-complex in a capillary, established the new method using cIEF, showing that can separate proteins within 5 minutes in hundreds-nano litter scale. In the regenerative medicine, this simple and easy technique might enable the standardization of iPS cells even from its primary colony.

Poster Board Number: F-3276

GENERATION OF A NOVEL NON-TRANSMISSIBLE AND CYTOSOL-REPLICATING RNA VIRUS VECTOR THAT ENCODES FIVE IPS CELL-INDUCING GENES AND A REPORTER GENE

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Reprogramming of somatic cells to induced pluripotent stem (iPS) cells can be achieved by the delivery of a combination of specific transcription factors. Although approaches using retroviral and lentiviral vectors are efficient and reproducible in generating iPS cells, they have a risk of damaging the host genome. In the present study, we generated a novel measles virus (MeV)-derived vector that encodes five iPS cell-inducing genes (Klf4, Oct3, Sox2, Glis1, and Pin1) and a fluorescent protein gene (EGFP). MeV is a member of the family Paramyxoviridae and possesses a nonsegmented negative-sense RNA genome. MeV replicates entirely in the cytosol without DNA stage, and, in principal, has no risk of damaging the host genome. Although, originally MeV has a nonsegmented RNA genome, we previously generated a MeV-derived vector possessing a segmented RNA genome that can harbor multiple additional foreign genes (Takeda et al. 2006 J Virol). The vector generated in the present study possessed a genome of two RNA segments. One genome segment encoded three MeV genes (N, P, and M) and three iPS cell-inducing genes (Klf4, Oct3, and Glis1). The other genome segment encoded two MeV genes (H and L), two iPS cell-inducing genes (Sox2 and Pin1), and a reporter protein (EGFP) gene. In addition, the genome of the vector was designed to lack the MeV F gene, and the F protein was provided in trans using a F protein-expressing cell line, making the vector safe, non-transmissible, and less cytopathic. Furthermore, we modified the H gene to make the vector highly infectious to human fibroblast cells. More importantly, neither helper virus nor complicated procedure was required to generate the vector. All required techniques were plasmid-transfection and culturing cells. In summary, the vector reported here is (1) safe for the host genome, (2) non-transmissible, (3) less cytopathic, (4) tolerant for accommodating six additional foreign genes, including five iPS cell-inducing genes, and (4) easy to generate. We are convinced that this MeV-derived vector has a great potential in using for regeneration medicine and gene therapy.

Poster Board Number: F-3277

STEM CELL IMAGING PROBE DEVELOPMENT BY DIVERSITY ORIENTED FLUORESCENCE LIBRARY APPROACH (DOFLA)

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Various types of stem cells at different stages of differentiation have been used for basic research and clinical applications. Recent success of somatic cell reprogramming into pluripotent or multipotent stem cells has made it possible to generate disease-specific cell culture models which are invaluable tools for understanding

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disease mechanisms and for drug developments. Typically, the types and differentiation stages of stem cells have been identified by genetic reporter system or immunocytochemistry which require genetic manipulations or binding of xenogenic antibodies. Therefore, the development of more convenient and safe methods to detect specific cells is a highly unmet need for both basic research and clinical applications of stem cells. We have developed diversity oriented fluorescence libraries (DOFL) composed of more than 10,000 intrinsically fluorescent small molecules by combinatorial chemistry. By high throughput screening of DOFL in various screening platforms, we have previously developed sensors and imaging probes for DNA, RNA, GTP, albumin, glutathione, heparin and beta amyloid to name a few. As an expansion of high throughput DOFL screening, we established live cell microscope image-based and flow-cytometry based high content screening systems to develop stem cell type- and differentiation stage-specific colorful chemical imaging probes. Detail examples and applications of the stem cell probes will be further discussed.

Poster Board Number: F-3278

GENERATION OF A GROWTH CURVE FOR IPS CELLS IN A FEEDER-FREE CULTURE BY NONINVASIVE IMAGE ANALYSIS

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Maintaining the undifferentiated state of iPS cells is an essential process in their culturing. However, the process requires improvement in the following three areas: 1. Feeder-free culturing: To eliminate variable factors in culturing, and to reduce the culturing cost. 2. Automation: To reduce the culturing cost, and to achieve consistency in the supply of iPS cells. 3. Establishment of a quantitative index: To quantify the culturing status, and to provide a clear index for evaluation. As a means for implementing these improvements, a system has been developed for time-lapse observation of a feeder-free culture, with which iPS colonies can be extracted from the observation images to provide the operator with information on changes in the colony areas. In constructing the system, Nikon BioStation CT cell culture observation system was used to capture time-lapse images of the entire surface of a 6 well-plate (100 images with a 2x objective (4x4mm)) over a one-week period of feeder-free culturing (using ReproCELL ReproFF2), from seeding to passage. Once a culture dish is inserted and an observation schedule is set, the system automatically captures images while maintaining the observation position across daily medium replacements, allowing the growth of each colony to be tracked with ease. CL-Quant image analysis software was used for teaching of the iPS colonies based on the captured time-lapse images, thus allowing iPS colonies to be identified within phase-contrast images, and changes in the colony areas to be plotted as a cell growth curve. The system was used to generate the growth curve for iPS cells cultured over a one-week period (from seeding to passaging) while maintaining the undifferentiated state. The growth curve was represented by a straight line, expressing an increase in the number of cultured cells. In conclusion, the system may be used with feeder-free culturing to automatically plot changes in the culturing status as a growth curve, thereby establishing an objective evaluation index.

Poster Board Number: F-3279

EXAMINING BRAIN EVOLUTION BY CELLULAR REPROGRAMMING

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Despite their substantial genomic similarity, humans and chimpanzees differ considerably in terms of brain function, cultural complexity and language acquisition. Because there is little evidence that simple addition or deletion of genes is sufficient to explain such differences, changes in the regulation of genes shared between humans and chimpanzees have been proposed to play an important role in shaping neuronal networks and perhaps defining cognitive differences between the two species. All the information available to date for comparative studies between humans and our closest relatives come from DNA/RNA samples extracted from preserved (post-mortem) tissues. These samples may not accurately represent the distinctive traits of live cell behavior. Pluripotent stem cells can recapitulate early stages of an organism's development that may cause the phenotypic differences. In this study, we characterize induced pluripotent stem cells (iPSC) derived from somatic cells from humans, bonobos and chimpanzees. Non-human primate iPSC behave similarly to human cells regarding self-renew capacity and pluripotency potential. Moreover, iPSC-derived mature neurons from all species were able to express basic properties of functioning neurons. Importantly, we demonstrate that non-human primates' cells have a different gene expression profile and behave differently in culture, when compared to human cells. We propose that by comparing the development of live human neurons with our closest relatives we may be able to bring new insights to the molecular mechanisms and phenotypical traits involved in the specification of human brain. This approach provides a unique biological resource to elucidate the phenotypic differences between human and apes at precise time points during cellular specification.

Poster Board Number: F-3280

CHARACTERIZATION OF DYSGERMINOMA LIKE TUMORS AROSE IN THE PROCESS OF GENERATING COMMON MARMOSET INDUCED PLURIPOTENT STEM CELLS

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The development of technology to generate induced pluripotent stem cells (iPSCs) have brought us a solution to overcome the ethical and immune rejection problems with embryonic stem cells (ESCs) as a cell source for human regenerative medicine. However iPSCs are generated by the transduction of transcription factors including oncogenes such as c-MYC and KLF4, a risk of a possible tumor formation originated from the functional cells transplanted into patients remains to be solved. Thus it is crucial to characterize iPSCs using an appropriate animal model. The Common Marmoset (CM, callithrix jacchus) is considered to be a very useful animal model for preclinical testing of new therapeutic strategy for various

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diseases mainly because of their genetic similarity to humans. To generate iPSCs from CM cells, we have lentivirally transduced reprogramming factors including OCT3/4, SOX2, KLF4 and c-MYC into CM fibroblasts derived from aorta-gonad-mesonephros (AGM) area. We found the formation of several round-shaped colonies expressing ES markers such as TRA1-60, SSEA4 and LIN28. However these colonies represent abnormal karyotype denoted as 46, X, del(4q), +mar. As the karyotype of parental cell line, AGM fibroblasts was 46, X, + mar, chromosome 4q have been thought to be lost in the process of reprogramming. We called the round-shape colony forming cells as abnormally reprogrammed cells (ARCs). Because important tumor suppressor gene such as tumor suppressor gene locus 1 (TSG1), large tumor suppressor 1 (LATS1) and P36 transformed follicular lymphoma gene (P36TFL) locate chromosome 4q, ARCs might have been transformed. Indeed ARCs form a tumor when they were injected into severe combined immune deficiency (SCID) mice, and tumor was found six weeks after the injection. Immunohistochemical analyses revealed that the tumor was negative for several differentiation makers but positive for c-KIT, and histological examination revealed that the tumor was very similar to human dysgerminoma. To further characterize these dysgerminoma like tumors (CM DGs), we generated five different CM DG cell lines, and found that CM DGs could be cultured in a semifloating condition in 10%FBS/DMEM-F12. We detected the expression of endogenous and exogenous reprogramming factors including OCT3/4, SOX2, KLF4 and c-MYC. It is possible that undesirable tumors may accidentally be formed in patients with various diseases who received iPSC-based therapies. To collect information for the future possible treatment of such tumors, we examined the effect of DNA damaging agents (mitomycin-C and cisplatin) and irradiation on CM DGs, and found that DNA damage agents and irradiation effectively inhibit the growth of CM DGs by the induction of cell death. We also found that knockdown of SOX2 by shRNA remarkably inhibited the proliferation of CM DGs, indicating that the growth of CM DGs was dependent on SOX2 expression. Taken together, our study indicates that DNA damaging agents, irradiation and molecular targeted therapy downregulating reprogramming factors are effective treatments for unexpected tumors derived from transplanted cells derived from iPSCs.

Poster Board Number: F-3281

IN VITRO INTERSPECIES CHIMERA ASSAY FOR PLURIPOTENT STEM CELL

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One of the emerging problems in characterizing pluripotent stem cells is the limitation of functional assays. To evaluate pluripotentiality of non-rodent pluripotent stem cells, differentiation competence into all three germ layers is only tested such as teratoma formation or *in vitro* differentiation, though chimera-formation is the gold standard characteristics for rodent pluripotent stem cells. It is known that human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) have more characteristics in common with mouse EpiSCs than rodent ESCs/iPSCs. As mouse EpiSCs are non-chimera-forming pluripotent stem cell lines, human ESCs/iPSCs are also expected not to contribute to chimera. Due to ethical and technical difficulties, however, chimera-forming ability has not been tested for human ESCs/iPSCs. To solve this problem, we developed an *in vitro* chimera assay for human pluripotent stem cell lines. Following the guidelines for human ESCs in Japan, we designed the assay to use mouse pre-implantation embryos to make interspecies chimera with human iPSCs, and cultured *in vitro* up to the early epiblast. Injected hiPSCs were lost in chimeric embryos day by day, and completely disappeared within 7 days *in vitro*. This suggests that hiPSC could not contribute to development of mouse pre-implantation embryo, consistent with previous reports. Therefore, we propose this *in vitro* interspecies chimera assay as useful to characterize developmental plasticity of human or other animals pluripotent stem cells.

Poster Board Number: F-3282

INFERING THE TRANSCRIPTION NETWORKS REQUIRED FOR INDUCTION OF PLURIPOTECY

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Somatic cells can be reprogrammed into induced pluripotent stem (iPS) cells by introducing a few transcription factors. However, only a minority of original somatic cells becomes iPS cells and it takes long period to establish them. These facts indicate that stochastically rare events after transgene expression and/or particular intrinsic properties of donor somatic cells are required to achieve pluripotency. To elucidate the molecular mechanisms that can direct the somatic cells toward reprogramming, we profiled the gene expression in mouse embryonic fibroblast (MEF) at the early stages of iPS cell derivation. First, we investigated the timing at which the somatic cells are committed to be reprogrammed into iPS cells. Surprisingly, we found that the cell fates are determined by as early as three days after retroviral introduction of reprogramming factors. Second, by BioMark high-throughput real time PCR system, we compared the gene expression at the early stages (day 3, 6) in the two types of cells, one is efficiently reprogrammed at the later stage (day 12) and the other is not. As a result, we found that the ratio of the exogenous expression level of Oct4 to that of Sox2 at day3 is important for efficient reprogramming, and that the expression levels of several genes including some chromatin modifiers and some cell surface proteins at day 6 are positively correlated with reprogramming efficiency. By using FACS with antibodies for those cell surface proteins, we could enrich the cells expressing pluripotency genes such as Rex1 in the early stage of iPSC derivation. Although we identified several genes whose expression levels in the early stage positively correlate with reprogramming efficiency, only the expression of any single gene can convincingly predict whether somatic cells become iPS cells or not. Therefore, we hypothesized that iPS cell derivation requires multiple gene expressions regulated by different pathways. Now, we are trying to investigate those transcription networks that activate the somatic reprogramming by combining BioMark system with RNAi analysis.

Poster Board Number: F-3283

ANTAGONISM BETWEEN PLURIPOTENCY AND DIFFERENTIATION REGULATING TRANSCRIPTION FACTORS

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Induction of iPS cells (iPSC) is known to be an inefficient process. It has been shown that a number of genes, including p53 and Ink4a/Arf, act inhibitory to this process. In an attempt to enhance iPSC induction efficiency, in this study we sought to find another set of genes reducing iPSC induction efficiency. We focused on transcription factors (TFs), because of their potential to affect expression of large number of target. As a model cell type, we employed an artificial cell line (NSEB5-2C), which proliferates rapidly while

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maintaining as a pure population (i.e. suitable for experiments). NSEB5-2C exhibits three characteristics; i) Fgf2/Egf dependency for proliferation, ii) capability to differentiate into TuJ-positive cells, iii) neural progenitor/precursor-like transcriptional profile. To circumvent problems of knockdown efficiency and functional redundancy, which normally accompany with loss-of-function approach, we employed gain-of-function approach. The assay system of this study was that, at the same time with iPSC induction in NSEB5-2C, a single TF (already expressed in these cells) was overexpressed, and the resultant iPSC colony number was counted to give the index of the TF's effect (iPS interference assay). We assayed 160 TFs (we removed house-keeping TFs and known TFs involved in inhibition of iPSC induction), we found that TFs exhibited highest interference strongly maintained differentiation status; when overexpressed, they could induce NSEB5-2C-like cells carrying above three characteristics from an unrelated cell type (hepatoblasts). Similarly, when we used hepatoblasts for iPS interference assay, we observed the high interference in Foxa2 and Hnf4a, the master regulators of hepatic lineage cells. We propose that TFs maintaining cell-type-specific transcriptional profile may contribute to reduce the efficiency of iPSC induction.

Poster Board Number: F-3284

LIGANDS DEPENDENT AND INDEPENDENT EFFECTS OF RETINOIC ACID RECEPTORS (RARS) IN REPROGRAMMING MEFS TO IPSCS

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Somatic cells can be efficiently reprogrammed to pluripotent stem cells by introduction of Oct4, c-Myc, Klf4 and Sox2 (4F) with various methods. By combining the 4F with additional two factors, retinoic acid receptor gamma (RARg) and Liver receptor homolog-1(Lrh1) (6F), we are able to reprogramme both MEF and human fibroblasts to naïve state in the presence of the MEK and GSK-3 inhibitors (2i) and Lif using the *piggyBac* transposon system in a chemically defined medium. Using episomal vectors, expression of 6F, but not 4F, enables efficient production of vector-free naïve miPS cells. Vitamin A (retinol) is one of the components in this defined medium. Retinol can be metabolized into different retinoids to activate retinoic acid signalling by activating RARs. Compared to MEFs reprogrammed in the absence of retinol, the 4F reprogramming efficiency was increased in the presence of retinoids or RA agonists, and was suppressed by RA antagonists. We previously showed that overexpression of RARg and 4F dramatically increased reprogramming colony number. Addition of RA agonists in reprogramming medium can further increase the reprogramming colonies of Rarg plus 4F. On the other hand, 6F reprogramming is still much more efficient than 4F even in the absence of RA agonists. Genetically, the dominant negative forms of RARa (RARa-DN) or RARg (RARg-DN) blocks both 4F and 6F reprogramming. Adding RA agonists relieves this reprogramming blocking. Therefore, both ligand dependent and ligand independent mechanisms are involved in the 6F efficient reprogramming of MEFs to iPSCs.

Poster Board Number: F-3285

INDUCED PLURIPOTENT CELLS DERIVED FROM PERIPHERAL LYMPHOCYTES CAN BE DIFFERENTIATED INTO FUNCTIONAL NEURAL CELLS

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Human induced pluripotent stem cells (hiPSCs) derived from somatic cells of patients, are recognized as promising source for regenerative medicine and useful tool for disease modeling. Currently, most of hiPS clones are generated from dermal skin fibroblasts by inducing reprogramming factors. However, there are undeniable invasive risks of bleeding, infection, and scarring in skin biopsies. Although T-cell derived iPSCs (TiPS) (Seki et al., Cell Stem Cell, 2010) are perhaps the ideal cell source for patient-specific iPSCs due to the ease of obtaining patient samples, recent studies have indicated that iPS cells retain an epigenetic memory relating to their cell of origin that restricts their differentiation potential. To investigate whether TiPS have ability to generate functional neural cells as well as fibroblast-derived iPS cells and can be used for the study of neural diseases, we established both TiPS and fibroblast-derived iPS cells from the same patient by various method including Sendai virus (SeV), retrovirus and episomal vectors. While the efficiency of differentiation into neural lineage is significantly lower in TiPS, neural cells induced from TiPS were functionally similar to that from fibroblast-derived iPS cells. Therefore, we concluded that TiPS could be used as tools to study neural diseases.

Poster Board Number: F-3286

INDUCED PLURIPOTENT STEM CELLS OF THE COMMON MARMOSET MONKEY DISPLAY ESC-LIKE PLASTICITY

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Objective: Regenerative medicine is in need of solid and reliable human-like animal models for research of human diseases. Efficiency and clinical safety of embryonic stem cells (ESCs) and reprogrammed cell types in cell therapy and drug development have to be evaluated. The common marmoset (Callithrix jacchus) is an excellent link between rodents and human and readily utilized in biomedicine since decades. To minimize ethical concerns for using ESCs, induced pluripotent stem (iPS) cells could be an alternative source for such cell therapies. However, each reprogramming method has its own risks like impaired reprogramming transcription factor silencing, insertial mutagenesis etc. Only 2 attempts for reprogramming have been published so far, with variable success. In order to establish a solid and practicable protocol for reprogramming of marmoset cells, we produced iPS from A) a cell type easy to obtain with an B) excisable lentiviral vector system with a C) SFFV promoter known for easy silencing and D) containing only four reprogramming transcription factors Oct3/4, KLF4, Sox2 and c-Myc.

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Material and Methods: For reprogramming bone-marrow derived mesenchymal cells of the common marmoset were cultured. In the presence of TAV, SB431542, PD0325901 and ascorbic acid, the cells were transduced with a lentiviral SFFV driven quad-cistronic vector system in mTESR medium. The cells were cultured on matrigel and formed colonies were transferred onto mouse embryonic feeder cells after 21 days. Results: The cells obtained showed typical ESC-like morphology and were positive for alkaline phosphatase and the endogenous pluripotency markers Oct3/4, Nanog, Sox2, KLF4 and MYC while exogenous genes were downregulated. From passage 19 on cells were differentiated successfully to embryoid bodies (EBs). The injection of iPS cells into the kidney capsule of NODscid mice showed a teratoma formation after eight weeks. All three germ layers could be detected in EBs as well as in the teratoma. Also differentiation into neuronal progenitors, adipocytes, chondrocytes and osteogenic cells displaying all marker genes and morphology could be achieved. Conclusions: These cells appear to be the first reprogrammed iPS cells from the common marmoset with excised reprogramming vector. They display all pluripotency markers and display a plasticity similar to marmoset ESCs, which is an important step solid production of clinically safe pluripotent cells in this animal model.

Poster Board Number: F-3287

NEURAL DIFFERENTIATION FROM PRIMATE INDUCED PLURIPOTENT STEM CELLS DESIGNED FOR AN AUTO-GRAFTING MODEL SYSTEM.

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[Background] Our goal is the clinical application of induced pluripotent stem cells (iPSCs) to the cell therapy for Parkinson's disease (PD). To reduce the host vs. graft reaction minimally, it is hoped to prepare either self-iPSCs (iPSCs originated from a patient's own cells) or HLA-matched iPSCs. On the other hand, it remains uncertain to which extent the HLA-mismatched cells cause immuno-reaction in the brain or if the auto-grafting can really overcome those undesired phenomena. We are establishing the model system of auto-transplantation and allo-transplantation using cynomolgus monkeys. We succeeded in establishing primate iPSCs from normal cynomolgus monkey's fibroblasts. [Materials & Methods] We took fibroblasts from two cynomolgus monkeys, transfected six genes retrovirally and established the primate iPS cell lines. For neural induction we used the floating aggregation method (SFEBq): starting with the single dissociation of the undifferentiated iPSCs and culturing them in a 96-well plate with Lipidure-coated surface. [Results] We established and selected iPSC clones from two different monkeys. If the iPSCs were transplanted into a testis of aNOD-SCID mouse, they formed a teratoma composed of tissues from all three germ layers. Although the silencing of the transgenes was incomplete comparing to the case of human iPSCs, the primate iPSCs could differentiate into neurons responding well to our induction system. The iPSCs could differentiate into neurons including dopaminergic neurons that could survive in a brain of a NOD-SCID mouse after transplantation without forming tumor. [Future plan] We will transplant the iPSCs- derived neurons back to the brains of the original monkeys and evaluate the immuno-reaction.

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INDUCTION OF GROUND STATE PLURIPOTENCY FROM MAMMALIAN SOMATIC CELLS

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iPSCs have a great potential for regenerative medicine, however, preclinical studies using large animals are necessary before the application of iPSCs to human. Although disease models in large animals were expected to be essential for preclinical studies, iPSC lines that can maintain pluripotency without the support of exogenous factors and that can contribute to germ line chimera are not yet available for non-rodent mammalian species. We hypothesized that forced expression of four reprogramming factors, Oct3/4, Sox2, Klf4 and c-Myc, in somatic cells was sufficient to excite pre-pluripotent state and that culture environments during reprogramming process determined characters of the reprogrammed cells. Therefore, the iPS technology can be used to randomly and systematically screen the novel culture conditions. In this study, we tested various culture conditions to induce novel-type pluripotent stem cells, iGPSCs (induced ground state pluripotent stem cells, putative) from porcine fetal fibroblasts and identified conditions to generate iGPSCs. The iGPSCs were generated in the optimized culture condition by a doxycycline-inducible reprogramming system and an Oct3/4 reporter selection. These cells showed mouse ESC-like morphology and were positive for pluripotent markers. We are now verifying whether these conditions can induce authentic ground state of pluripotency in various mammalian species.

Poster Board Number: F-3289

DEVELOPMENT OF AN EFFICIENT AND VERSATILE HERPES SIMPLEX VIRUS VECTOR SYSTEM FOR CELLULAR REPROGRAMMING

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Human induced pluripotent stem (iPS) cells hold great promise for studies of genetic diseases and may prove useful for regenerative medicine applications. Yamanaka and colleagues first reported that expression of four transcription factors, OCT4, KLF4, SOX2 and c-MYC, is sufficient to reprogram human somatic cells to the pluripotent state. Other factors (e.g. Lin28, NANOG and GLIS1) can improve efficiency but a concern is that the reprogramming genes were introduced with genome modifying integrating retroviruses. To overcome this limitation, transient methods for reprogramming have been established that include transfection of mRNA or introduction of polypeptides, but reprogramming remained quite inefficient and required long-term culture. Our goal is to develop an improved reprogramming vector capable of expressing multiple reprogramming functions simultaneously. This vector must show high transduction efficiency without toxicity or integration into the host genome. Here we report the development and application of a high capacity herpes simplex virus (HSV) vector system that meets these criteria. Our HSV vectors encode the Yamanaka factors [OCT4, KLF4 and SOX2 (OKS)] in a polycistronic cassette or contain an expression cassette for the mir-302/367 cluster. In addition, they contain lambda phage attR recombination sites in the intergenic UL3-UL4 region to allow the rapid introduction of reprogramming-

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related factors such as MYC, dominant-negative p53 (p53DD) or GLIS1. The HSV vector is replication-defective and non-toxic due to deletion or altered expression kinetics of the five viral immediate early (IE) genes. Vector construction is carried out using bacterial and *in vitro* recombination systems and vector genomes are maintained as bacterial artificial chromosomes. Recombinant vector DNA isolated from bacteria is used to produce recombinant virus particles by transfection of complementing U2OS cells engineered to express a single missing IE gene in trans. The vectors also contain an mCherry expression cassette to visualize virus infection. Expression of the multiple transgene inserts is under transcriptional control of constitutively active promoters that include the chicken β-actin/HCMV (CAG) promoter/enhancer, the human elongation factor-1α promoter and the human ubiquitin C promoter. On infection of human neonatal dermal fibroblasts with our HSV-based OKS plus MYC (OKSM) or OKS plus p53DD (OKSp53DD) vectors, nearly 100% of the cells were strongly mCherry positive and the cultures abundantly expressed all of the OKSM or OKSp53DD reprogramming gene products. Experiments are in progress to test reprogramming efficiency of human fibroblasts. Taken together, our "hit-and-run" vector system provides the opportunity to study the mechanism of cell reprogramming without permanent genetic modification of the target cell.

Poster Board Number: F-3290

TRACKING IPS CELLS-INDUCED NEURAL PROGENITOR CELLS IN CNS OF RATS AND MONKEYS

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Background: Despite of immense breakthroughs of induced pluripotent stem cells, clinical application of iPS cells therapy remains hampered by a lack of definitive *in vivo* studies. Here, we attempted to track iPS cells-induced neural progenitor cells in the primate and rodent's central nervous system. Materials and Methods: 1. Induced pluripotent stem cells were generated from human fibroblasts with written informed consent. Then multistage differentiation protocol was used to induce the neural differentiation in vitro. 2. NPCs were labeled with green fluorescent protein, Feridex and protamine sulfate, and tested for cell proliferation, differentiation as well as electrophysiological behavior. 3. Traumatic brain injury rats were prepared. One week later, in group 1, human NPCs labeled with SPIO were injected around the brain lesion. Group 2 rats didn't receive cell implantation. Group 3 rats received phosphate buffered saline treatment. 4. Monkeys with spinal cord injury were prepared, and at one week post-injury, monkeys in group 1 received human NPCs labeled with SPIO injection. Monkeys in group 2 didn't receive cell implantation. Group 3 monkeys received PBS injection. 5. MRI scanning was performed with clinical 3.0T systems, and Gradient reflection echo sequence scanning was applied. The MRI was performed at 1, 3, 7, 14, 21, and 30 days following NPCs transplantation. 6. All rat brain sections were double stained by hematoxylin-eosin and Prussian blue to detect intracellular iron oxide particles. And immunohistochemistry was performed to detect the transplanted NPCs and their phenotypic features. Results: 1. Reprogrammed human iPS cells exhibited similar properties to human embryonic stem cells in morphology and properties. The induced neural cells showed typical neuronal cells morphology and expressed neuronal progenitor cells marker protein Marsh-1 and PSA-NCAM. 2. SPIO was detected in the cytoplasm as determined by Prussian blue staining. SPIO-labeled and unlabeled NPCs showed almost same cell viability. No significant difference was observed in the proliferation rate between them. 3. In group 1 rats, the injection sites were visible as areas of dark tissue following NPCs implantation. About two weeks later, the hypointense signals at the injection site faded a little and directed to the lesion site. The signals arround the lesion intensified during the third and fourth weeks after cell administration. The signals from MRI scan were in accordance with the results from rat brain sections with Prussian blue staining. In control groups, we did not observe these signal changes. 4. The injection sites were visible as circular areas of dark tissue after cell implantation in monkeys. About one to two weeks later, the hypointense signals faded a little around the injection site and extended to the lesion site. The hypointense signals around the lesion site intensified one month after NPCs injection. We did not observe obvious signal change in control groups. 5. The immunohistochemistry results showed that β-tubulin and GFAP-positive cells were found in the lesion sites. These data indicated that the implanted NPCs could not only migrate to lesion site, but also can differentiate into functional neural cells. Conclusions: In this study, we tracked iPS cells-induced neural progenitor cells migration in the rodent and primate CNS for the first time. These data provide necessary foundation for future clinical application of iPS cells.

Poster Board Number: F-3291

GENERATION OF IPS CELLS FROM NAKED MOLE-RAT, SENESCENCE AND CANCER RESISTANT ANIMAL

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Naked mole-rat (NMR, Heterocephalus glaber) is one of the only two eusocial mammals, like ant or bee. They live in a large colony consisting of a single breeding queen, king and many sterile castes such as solders and workers. The bleeding queen suppresses subordinate female's sexual maturity. NMR has been also received attention for their extraordinarily longevity. Surprisingly, NMR's maximum lifespan exceeds 30 years although their body size is same of mouse. The lack of an age-related increase in mortality rate indicates their negligible senescence. Moreover, these animals have never been observed to develop any spontaneous neoplasm.Therefore, NMR is regarded as "senescence-resistant" and 'cancer-resistant' animal model. Additionally, NMR also has some other features such as insensitivity to low oxygen, resistance to reactive oxygen species, unusual thermogenesis and insensitivity to certain types of pain. Considering these interesting characteristics, NMR would be a unique model for several biological and biomedical researches. Toward the molecular biological analyses of NMR's characteristics, the establishment of gene manipulation methods is essential. In mice, embryonic stem (ES) cell-mediated gene transfer technology is a powerful tool for the production of knockout or transgenic animals. ES cells are also useful to obtain various somatic cell types *in vitro*. However, NMR's low pregnancy rate and their specific sexual

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maturity system described above make it difficult to establish NMR ES cells. Thus, we have focused on the generation of induced pluripotent stem (iPS) cells from NMR's somatic cells. Recently, several groups have generated iPS cells from various mammalian somatic cells by transduction of defined transcription factors. Generation of iPS cells would overcome the difficulty in gene transfer in NMR. We introduced four factors (Oct3/4, Sox2, cMyc and Klf4) to NMR skin fibroblasts with ecotropic retrovirus. Seven days after transduction, the cells were harvested by trypsinization and plated onto feeder cells. The next day, the medium was replaced with a medium for ES cell culture condition. Three weeks after transduction, many ES-like morphology colonies had been appeared. The selected colonies were expanded and maintained on feeder cells. These colonies were alkaline phosphatase-positive. Reverse transcription-PCR showed that these NMR iPS cells expressed undifferentiated markers. Embryoid body formation showed that NMR iPS cells had the potential to differentiate into various somatic cell types. Now we are trying to characterize the NMR iPS cells in detail. Toward the discovery of genes play a critical role in NMR's unique characteristics, NMR iPS cells would be a convenient cell source and become a powerful tool for transgenic approaches in the future.

Poster Board Number: F-3292

INDUCED PLURIPOTENT STEM CELLS MAINTAINED PLURIPOTENCY MARKERS WITH ACTIVIN A IN FEEDER FREE CULTURE

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Introduction: Induced pluripotent stem (iPS) cells are promising cell source for regererative medicine. iPS cells are desirably free of proteins from other species. Mouse fibroblasts are used for feeder cells for human iPS cells. iPS cells are not suitable for transplantation when cultured on mouse feeder cells since they are exposed to mouse proteins. Culture media are available for feeder free culture of iPS cells. These media sometimes fail to maintain pluripotency of iPS cells. Moreover, ingredients of the media are not informed. No media is available for feeder free culture of iPS cells, or the ingredients are known. Activin A is used to promote pluripotent cells to differentiate to endodermal cells. We added Activin A in embryoid bodies for human iPS cells to differentiate to hepatocytes. Unexpectedly, iPS cells did not change morphology. They were positive for ALP staining, Oct3/4, Nanog, SSEA4 and TRA-1-60. Our previous report indicates that Activin A maintains pluripotency of iPS cells. Our next step was to investigate the passage of iPS cells with Activin A. Materials and Methods: Human iPS cells (201B7, RIKEN Cell Bank) were cultured on 6-well plated coated with Matrigel in media of Dulbecco's Minimum Essential Medium-F12 medium supplemented with 20% of Knockout Serum Replacement, 10% of Minimum Essential Amino Acids (Invitrogen), 2 mM of L-Glutamine (Invitrogen) and 1 mM of 2-Mecaptoethanol (iPSm(-)). The added reagents were SU5402 (FGFR1 inhibitor) at 2 μM, SC-1 (RasGAP, ERK1 inhibitor) at 1 μM, Activin A at 10 ng/ml, CHIR99021 (GSK-3 beta inhibitor) at 2 μM, human leukenia inhibitory factor (LIF) at 1000 U/ml, or basic fibroblast growth factor at 5 ng/ml. Culture of iPS cells was completed when they differentiated, disappeared, or reached 12 passages in the media above mentioned. Alkaline phosphatase (ALP) staining was performed on the iPS cells in 6-well plates. The iPS cells were immunostained in 4-well chamber slides with antibodies against Oct3/4, Nanog, SSEA4, and TRA-1-60. One factor analysis of variance was used for statistical analysis (JMP 8.0). Results: iPS cells were damaged after one passage with SU5402 or SC-1. The experiments were continued with the other reagents. Average passage number was 9.25 ± 2.76 and 4.00 ± 1.41 in media with or without Activin A, respectively (P=0.0003). iPS cells in Activin A (A), Activin A and CHIR99021 (AC), and Activini A, CHIR99021 and LIF (ACL) reached 12 passage. iPS cells in A, AC, and ACL were positive for ALP staining in media with (ACL) at 12 passage. iPS cells were positive for Oct3/4, Nanog, SSEA4, and TRA-1-60 at 12 passage. The signals were relatively weaker of SSEA4 and TRA-1-60 of iPS cells in ACL than those in A or AC. Discussion: Our results clearly showed that Activin A significantly prolonged passage number maintaining pluripotency. Additionaly, C or L helped iPS cells remain pluripotent in conjunction with Activin A although each did not extend passage as single reagent. The results of SSEA4 and TRA-1-60 suggested that C was more preferable reagent with Activin A than L. In conclusion, Activin A was one of the ingredients of media for feeder free culture of human iPS cells.

Poster Board Number: F-3293

EFFECT OF ES CELL FACTOR AND PROTO-ONCOGENE HMGA2 ON FORMATION AND DIFFERENTIATION OF IPSC

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HMGA2 is an oncofetal, non histone chromatin factor generally expressed in pluripotent cells and during early developmental stages. Abnormal expression of HMGA2 is seen in most human cancer cells (regulated by the Lin28/let7-miRNA pathway). HMGA2 has been linked to body height and size in humans, dogs, mice and chicken, with type 2 diabetes, lipoma formation, metastasis, uterine leiomyomas and, based on work carried out in our laboratory, with ESC genome repair and stability. HMGA2 is expressed in neural stem cells and is essential for normal heart development in Xenopus leavis. Here we show that iPSC over-expressing HMGA2 during iPSC induction formed indistinguishable colonies to the control, although colony numbers decreased. Resulting HMGA2-iPSCs were characterized for pluripotency by OCT4, AP, SSEA1 expression and EB formation and showed to be similar to control colonies. However an increase in Nestin expression was seen in HMGA2 overexpressing cells following EB formation, suggesting these cells may favor ectodermal differentiation. Upon directed differentiation to cardiomyocytes, HMGA2 cells showed less potential to generate beating cardiomyocytes. This implies that HMGA2 may be regulating specific developmental pathways during differentiation. Whole transcriptome analysis of these cells led to interesting insights into HMGA2 functions and biological processes which are highly relevant to the known phenotypes linked to aberrant HMGA2 expression. Our results reveal a striking example of the usefulness of iPSC in elucidating the molecular functions of genes known to be associated with certain developmental phenotypes.

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Poster Board Number: F-3294

TOWARDS A NON IMMUNOGENIC GENE DELIVERY VECTOR SYSTEM USING HUMAN ARTIFICIAL CHROMOSOME FOR GENE AND CELL THERAPY

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Human artificial chromosomes (HACs) have several advantages for gene- and cell-therapy, i.e., non-integration and no size limitation of transgene. For an example, we have shown that HACs can carry and correct a 2.4Mb genomic DNA for the DMD gene therapy. In order to prove the concept toward gene- and cell- therapy first in a mouse model, we constructed a mouse artificial chromosome vector MAC1 from a mouse chromosome 11. This MAC1 is maintained independently from host chromosomes, with a stable maintenance and gene-expression in cultured cells and mice. However, the method requires a selective marker gene for transgene maintenance or fluorescent labeling of cells. Most importantly, marker genes may activate immune response. To avoid the potential risk, a novel system to remove marker genes is required. Here, we reconstructed a non-immunogenic MAC(ni-MAC) for gene delivery, which has a system with FLPe-FRT recombination to remove selective and fluorescence marker genes that are reported as potential immunogens and a loxP site to introduce a gene of interest by chromosome engineering technology using chicken pre-B cells (DT40) and CHO cells. In this model system, we introduced RFP as a substitute of therapeutic gene {ni-MAC(RFP)}. Then, ni-MAC(RFP) was transferred to mouse ES cells by microcell-mediated chromosome transfer ,and that was followed by transient expression of FLPe in mouse ES cells. We could obtain clones expressing RFP and holding ni-MAC without marker genes from mouse ES cells as well as CHO cells. We confirmed that MACs were constructed correctly and maintained independently from host genome by PCR and FISH analyses. We will investigate the immune response of this gene delivery system *in vivo* and vitro and compare with several conventional gene delivery methods.

Poster Board Number: F-3295

IN VITRO DIFFERENTIATION POTENTIAL OF PORCINE INDUCED PLURIPOTENT STEM CELL-LIKE CELLS DERIVED FROM NEURAL PROGENITOR CELLS

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Induced pluripotent stem cells (iPSCs) represent an innovative and promising tool to study human genetic diseases. The pig has the potential to become an important large animal model within translational biomedical research, particularly for the preclinical testing of iPSC cell therapies. However, the generation and characterization of porcine iPSCs (piPSCs) is hampered by a lack of full iPSC reprogramming including activation of endogenous pluripotency genes and silencing of the reprogramming transgenes. In this study, we characterized piPSC-like cells from porcine epiblast-derived neural progenitor cells (NPCs; Rasmussen et al., 2011) by use of a tetracycline-inducible Tet-ON approach. The piPSC-like lines were generated by use of a polycistronic lentiviral vector containing the porcine pluripotency genes: pOCT4, pSOX2, pc-MYC, and pKLF4 (pOSMK). Two piPSC-like lines were cultured in iPSC medium containing DMEM/F12, 20% KSR, 1% NEAA, 10μM β-Me, 1% Pen/strep, 20ng/ml human bFGF and 2μg/ml doxycycline for more than 30 passages. IPSC-like colonies were fixed with 4% PFA and processed for immunocytochemistry (ICC). To evaluate the differentiation capabilities, both iPSC-like lines were subjected to embryoid body formation in the differentiation medium (iPSC medium without bFGF and doxycycline) for 4 days in hanging drops and were then allowed to aggregate in suspension for an additional 3 days. EBs were fixed with 4% PFA and processed for cryosectioning and ICC for localization of the differentiation markers TUJ1, SMA, AFP, Vimentin, Cytokeratin, and Nestin, as well as OCT4. Furthermore, ten EBs per well were plated onto 0.1% gelatin-coating plastic coverslips and cultured in differentiation medium supplemented with 10mM ascorbic acid. At day 14 and 21, differentiated cells were fixed with 4% PFA and processed for ICC analyses with the differentiation markers TUJ1, SMA, and AFP as well as OCT4. Before differentiation, both piPSC lines expressed the pluripotency markers OCT4, NANOG, TRA-1-81, SSEA-4, and alkaline phosphatase, whereas, only one line was positive for SSEA-1. The sectioned and plated EBs were positive for markers of all germ layers, including endoderm (AFP, Cytokeratin), mesoderm (SMA, Vimentin) and ectoderm (Nestin, TUJ and GFAP). In conclusion, the piPSC-like cells possessed the potential to *in vitro* differentiate into all three germ layers. Although more efforts are needed for directed differentiation into mature cell types to be achieved, these results are important for testing the safety and efficacy of iPSCs procedures in the pig prior to the initiation of human trials.

Poster Board Number: F-3296

INDUCED PLURIPOTENT MESENCHYMAL STROMAL CELL CLONES RETAIN DONOR-DERIVED DIFFERENCES IN DNA METHYLATION PROFILES

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Reprogramming of somatic cells into induced pluripotent stem cells (iPSC) is an epigenetic phenomenon. It has been suggested that iPSC retain some tissue-specific memory whereas little is known about inter-individual epigenetic variation of iPSC clones. In this study we have reprogrammed mesenchymal stromal cells (MSC) from human bone marrow by retrovirus-mediated overexpression of OCT-3/4, SOX2, c-MYC, and KLF4. Global DNA-methylation profiles of the initial MSC, MSC-derived iPSC (iP-MSC) and embryonic stem cells (ESC) were then compared using a high density DNA-methylation array covering more than 450,000 CpG sites. Overall, DNA-methylation patterns of iP-MSC and ESC were similar whereas some CpG sites revealed highly significant differences, which were not related to parental MSC. Furthermore, hypermethylation in iP-MSC versus ESC was particularly enriched in developmental genes as well as shore regions next to CpG islands indicating that these differences are not due to tissue-specific memory or random *de novo* methylation. Subsequently, we searched for CpG sites with donor-specific variation in MSC preparations. These "epigenetic fingerprints" were highly enriched in non-promoter regions and outside of CpG islands - and they were maintained

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upon reprogramming into iP-MSC. In conclusion, DNA methylation profiles of iP-MSC clones from the same donor were closely related despite heterogeneity of MSC. On the other hand, iP-MSC maintain donor-derived epigenetic differences. In the absence of isogenic controls for disease modeling applications, it would therefore be more appropriate to compare iPSC from different donors rather than a high number of different clones from the same patient.

Poster Board Number: F-3297

REPROGRAM CELL FATE USING RNA

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Several methods have been used to induce somatic cells to re-enter the pluripotent state. Viral transduction of reprogramming genes yields higher efficiency but involves random insertions of viral sequences into the human genome. Although induced pluripotent stem (iPS) cells can be obtained with the removable PiggyBac transposon system or an episomal system, both approaches still use DNA constructs so that resulting cell lines need to be thoroughly analyzed to confirm they are free of harmful genetic modification. Thus a method to change cell fate without using DNA will be very useful in regenerative medicine. To achieve this goal, we synthesized mRNAs encoding OCT4, SOX2, cMYC, KLF4 and LIN28 and transfected them into fibroblast cells. Upon transfection, fibroblasts rapidly expressed these factors at levels comparable to, or higher than those in embryonic stem (ES) cells. We found that transfecting fibroblasts with a mixture of mRNAs encoding all five factors significantly increased the expression of endogenous OCT4, NANOG, DNMT3b, REX1 and SALL4. When such transfected fibroblasts were also exposed to several small molecules and cultured in embryonic stem cell (ES) medium they formed small aggregates positive for alkaline phosphatase activity and OCT4 protein. Our results demonstrate that mRNA transfection can be a useful approach to precisely control the protein expression level and short-term expression of reprogramming factors is sufficient to activate pluripotency genes in differentiated cells.

Poster Board Number: F-3298

WHAT IS THE OPTIMAL COMBINATION OF POTENTIATORS OF IPSC INDUCTION?

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Many conventional iPSC induction methods suffer from relatively low reprogramming efficiency which necessitates a high number of starting cells. Establishment of patient-specific iPSC banks has created the need for more efficient reprogramming methods, allowing the use of smaller numbers of cells. One way to address this issue is to optimize reprogramming efficiency by supplementing the culture medium with small molecular inhibitors, resulting in a more economical reprogramming process. Our aim was to derive a method for easy assessment of the effect of different induction conditions and to further optimize the induction conditions by testing the combinatorial effects of different small molecular inhibitors, previously described in the literature to enhance iPSC induction efficiency. We used reprogrammable human skin fibroblast cell batches, infected with bi-cistronic retroviruses encoding OCT4+KLF4 and SOX2+c-Myc and frozen two days after the infection. Colony formation was assessed by alkaline phosphatase staining on day 17 of the fresh cells, or on day 15 after thawing of the frozen cells. There was no signifficant difference in the reprogramming efficiency, demonstrating that the reprogramming process can be interrupted by freezing, thus validating this approach for the optimization of induction conditions. To test for the effect of different inhibitors, combinations of selected small molecules were applied to the reprogramming medium from day 6 to day 17 of induction. The combination of HDAC inhibitor sodium butyrate with the TGF-beta inhibitor SB431542 was found to increase the induction efficiency 160 fold, and these inhibitors have thus been used used also for further rounds of optimization. Interestingly, additon of MEK inhibitor PD0325901 reduced the reprogramming efficiency when using retroviral vectors, but when applied to cells induced by Sendai viral vectors (CytoTune, Invitrogen) it had a positive effect on colony numbers. Our results show that iPSC derivation can be significantly enhanced by optimizing the conditions used. Furthermore, these conditions have to be adjusted for the type of method used for genetic transduction.

Poster Board Number: F-3299

AUTOMATED NON INVASIVE CULTURE AND EVALUATION SYSTEM FOR IPS CELLS UNDER NEURAL DIFFERENTIATION PROCESS

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Neurons derived from human iPS cells are promising sources for future basic research, therapeutic studies, and drug screening. Providing human neural cells with consistent quality is essential for these studies. However, the neural differentiation process is time- and resource- consuming. The process takes four to seven weeks and there are no easy ways to objectively evaluate differentiation quality. This can be problematic for quality control because the existing evaluation process is often highly dependent on the skills and experiences of the researcher. Furthermore, chemical fixation, which is frequently used for the evaluation process, renders the cells unusable for downstream processing specific to regenerative research purposes. Therefore, an automated and non-invasive culture and evaluation system has been in high demand. We aimed to develop an integrated system which enables quantitative and label-free analysis of cells being differentiated from iPS cells toward neurons, while keeping them within a stable environment. There are two morphological check points in the neural differentiation process: one is the "rosette" structure which appears during neuro-epithelial and the neuro-progenitor stages, and the other is the neurites seen at later stages. High yields of rosettes are known to indicate a good differentiation status (Hu and Xhang, 2010). It has been reported that cells forming rosettes can be subcultured while maintaining the potential of early neural stem cells (Elkabetz et al., 2008). Therefore, we developed two image analysis algorithms for the detection of these signature structures, one for the rosette structures and the other for neurites. IPS cells were differentiated toward several types of central-nervous system neurons. During the entire differentiation process, cells were cultured in BioStation CT (Nikon) in which phase-contrast images were captured automatically. To analyze neurite extension, cells at the rosette-exhibiting stage were collected, dissociated, and plated for further differentiation. The rosette-like structures detected by one of our developed algorithms showed 84 % match from those detected by skilled operators with a high correlation coefficient of 0.94. Neurites and cell bodies were also detected by the other newly-developed algorithm. The neurite elongation rate was high when many rosettes were formed. These label-free image analysis can provide quantitative values such as the amount of rosettes per well and the neurite

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elongation rate with respect to the proliferation rate of co-existing cells. Integration of an automated culture and observation system with quantitative image analysis can be utilized to evaluate the efficiency of differentiation and can also be used to make predictions before the various differentiation phases occur. Such a platform consisting of a stable culture environment, automated live-cell imaging, and predictive image analysis will become a strong tool for live-cell screening.

Poster Board Number: F-3300

GENERATING POTENTIALLY SAFER IPSCS WITH A HELPER PLASMID-FREE PIGGYBAC TRANSPOSON SYSTEM

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DNA Transposons have emerged as promising genetic tools for mammalian genomic manipulation. *piggyBac* transposon, isolated from the genome of cabbage looper moth, shows the advantages of high transposition efficiency and precise excision without destroying the host genome. These properties make *piggyBac* an ideal vector for generating transgene-free iPS cells. The most commonly used *piggyBac* system contains a donor plasmid consisting of transgene within *piggyBac* terminal repeats, and a helper plasmid encoding *piggyBac* transposase. This system, however, possesses some potential risks. First, the prolonged supply of *piggyBac* transposases by the helper plasmid in cells may introduce undesired consequences including multiple transgene integrations and the re-mobilization of the pre-existing transgene integrated by *piggyBac* in host genome. Second, reintroducing the *piggyBac* transposase containing helper plasmid back to iPSCs for removing the transgenes is likely to case damages to the cells. To overcome this drawback, here we aim to develop a novel *piggyBac*-based platform for manipulating the mammalian genome using a recombinant *piggyBac* transposase. To this end, we constructed a series of *piggyBac* recombinant constructs using pTriEx as a vector. Surprisingly, most of these constructs display much higher activities than the wild type *piggyBac* when evaluated as a helper plasmid form with up to an 8-fold increase for the highest one in some cell line tested. This observation indicates that the modification done on *piggyBac* transposase may enhance its enzyme activity, suggesting the potential of developing the *piggyBac*-based site-specific therapeutic gene delivery vector. The establishment of this recombinant transposase-based platform will simplify the transposon system and reduce the damage during manipulation which in turn maintains the genome stability of the host. Applying such a platform in generating transgene-free iPS will facilitate the process of advancing its clinical application in regeneration medicine.

Poster Board Number: F-3301

QUALITY CONTROL OF IPS CELLS BY UNIQUE GENE TRANSFER SYSTEM

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We have developed unique gene transfer system based on persistent mutant strain of Sendai virus. The vectors, named SeVdp vectors, remain persistently in the cytoplasm without integrating into the host genome and also enable transfer and expression of multiples genes, which can be removed by siRNA treatment for viral polymerase gene (Nishimura, K. et al. 2011). These properties make it exceptionally suitable for production of induced pluripotent stem (iPS) cells. Indeed, an SeVdp vector harboring four reprogramming factors (Oct4, Sox2, Klf4 and c-myc) can reprogram mouse and human somatic cells efficiently, ultimately establishing iPS cells that are free of any transgene. In this presentation, we report analyses of iPS cells induced by different SeVdp vectors which express the reprogramming factors with different expression balance. From SeVdp vector, transgenes are expressed with fixed balance from a single vector genome, and the balance can be altered by exchange of their location or insertion of another transgene in the genome. We prepared some vectors expressing the four factors with different expression balance and used them for cell reprogramming. Investigating ES cell-specific marker gene expression in the induced colonies by each vector showed that the difference of the expression balance caused different expression pattern of the marker genes in the iPS cells, although all vector expressed same four factors. Moreover, among them, some vectors only induce partially reprogrammed cells. These results indicated that the expression balance of reprogramming factors is important for the quality of iPS cells, and we can control the quality by using the suitable SeVdp vector that express the factors with defined balance. So, our vector system was useful not only for the establishment of transgene-free iPS cells, but also for analysis of the mechanism of cell reprogramming.

Poster Board Number: F-3302

DVELOPMENT OF ALL-IN-ONE INDUCIBLE LENTIVIRAL VECTOR FOR GENE SPECIFIC ANALYSIS OF REPROGRAMMING

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For fair comparison of reprogramming efficiencies and *in vitro* differentiation capabilities among induced pluripotent stem cell (iPSC) lines, we constructed a single cassette all-in-one inducible lentiviral vector (Ai-LV) for the expression of three reprogramming factors (Oct4, Klf4 and Sox2). To obtain multiple types of somatic cells having the same genetic background, we generated reprogrammable chimeric mice using iPSCs derived from Ai-LV infected somatic cells. Then, hepatic cells, hematopoietic cells and fibroblasts were isolated at different developmental stages from the chimeric mice and re-reprogrammed to generate 2nd iPSCs. Consequently, the reprogramming efficiency of fibroblasts was decreased as developmental stage progressed. Although only a few colonies were generated, similar results were observed in the case of hematopoietic cells. The most remarkable difference was observed in hepatic cells. Fetal hepatoblasts were reprogrammed 1200 times more efficiently than adult hepatocytes. However, we found that forced expression of c-Myc improved the reduced reprogramming efficiency in aged somatic cells without affecting cell proliferation. Taken together, all these findings suggest that the Ai-LV system enables us to generate a panel of iPSC clones derived from various tissues with the same genetic background, and thus provides an invaluable tool for iPSC research.

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Poster Board Number: F-3303

MOLECULAR BIOLOGY OF DIFFERENTIATION-RESISTANCE IN PLURIPOTENT STEM CELLS

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Differences between human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) are one of the controversial subjects although it has been well-studied. Such controversies could be sustained because small number of lines has been compared and it was difficult to discuss biological meaning of differential signatures. Here we examined gene expressions and DNA methylation patterns of 49 hiPSC and 10 hESC lines cultured under the same conditions. We did not identify molecular signatures (gene expression or DNA methylation) that clearly distinguish hiPSCs from hESCs. We also found that most iPSC clones are comparable to hESCs in neural differentiation by forming aggregates with inhibitors of BMP and TGFβ. However, we identified 6 iPSC clones that retain substantial amounts of OCT3/4 positive undifferentiated cells after neural differentiation. Through induction into dopaminergic neurons these six "bad" iPSC clones formed teratomas when transplanted into the brains of immune-deficient mice. We then examined subclones of these bad iPSC clones and found that some subclones had normal ability of neural differentiation, whereas the other subclones retained poor differentiation propensity. Comparison between good and bad clones, and between good and bad subclones, identified some genes those are upregulated in bad clones and subclones. Notably, we focused the promoter activated with irregular DNA hypomethylation and chromatin modification in bad clones and subclones. These data demonstrated that most iPSC clones are indistinguishable from ESCs in gene expression, DNA methylation, and neural differentiation, and that rare "bad" iPSC clones abnormalities can be generated by epigenetic alteration.

Poster Board Number: F-3304

NEGATIVE FEEDBACK REGULATION OF OCT-4 UPON DIFFERENTIAL DOSE OF NANOG IN P19 CELLS

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Oct-4 and Nanog have been widely used for generation of induced pluripotent stem cell (iPS cell). Generally, Nanog increases Oct-4 transcription; however, its expression is down-regulated by high-dose Nanog. It is generally known that there is negative feedback regulation for Oct-4. However, its precise mechanism remains elusive. Here, we generated *Oct-4* promoter-luciferase constructs (-2179, -1879, -1346, and -754) by well-known 4 conserve region (CR) of *Oct-4* promoter. Luciferase activities of Oct-4 were declined upon high-dose Nanog in all constructs. These decreased effects were rescued by DNMT and HDAC inhibitors (5-AZA-cytidine and trichostatin A, respectively) in 3 constructs using dual-luciferase assay. And, this inhibitor assay was also confirmed by semi-quantitative RT-PCR and Western blotting in P19 embryonic carcinoma cell. These results suggest that DNMT and HDAC modulate this negative feedback regulation of Oct-4 upon high-dose Nanog. To identify the precise position in Oct-4 promoter, ChIP assay was performed for CR1-4 regions. As a result, DNMT3A binds to CR1 region upon high-dose Nanog. In addition, HDAC2 more binds to CR3 region upon high-dose Nanog than low-dose Nanog. Taken together, these data imply that Oct-4 negative regulation upon high-dose Nanog is accomplished by DNMT3A and HDAC2 binding to CR1 and CR3, respectively. Further, it will be investigated that the other HDAC can regulate these regions. This is first time to analysis negative regulation of Oct-4 upon high-dose Nanog. These results further bring about a new tool for generation of iPS cells and embryonic stem (ES) cell regulation. [This study was supported by 2011 Post Doctoral Fellowship Program of National Institute of Animal Science, Rural Development Administration, Republic of Korea. This work received grant support from the Agenda Program (No. PJ007577), Rural Development Administration, Republic of KOREA.]

Poster Board Number: F-3305

DYNAMIC ALTERATION IN RNA SPLICING DURING SOMATIC CELL REPROGRAMMING

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Elucidating the molecular mechanisms underlying somatic cell reprogramming is important not only to realize the application of iPS cells to regenerative medicine but also to understand basic principles of biology. To approach this problem, we have been performing transcriptome analysis. Here we report our analysis on changes in alternative splicing during reprogramming. By combining deep sequencing with high-throughput quantitative RT-PCR, we revealed drastic changes in genome-wide splicing profiles during the reprogramming process. Our analysis identified more than 600 genes, whose splicing patterns were altered during iPS cell induction from mouse embryonic fibroblasts (MEFs). Computational analysis of primary sequences demonstrated that several motifs were enriched in sequences wihtin, and adjacent to, the differentially-expressed skipped-exons between MEFs and iPS cells. Moreover, we also found that the lengths of the introns around exons, which are preferentially included in iPS cells, tend to be shorter. We further characterized the splicing patterns of the genes we identified. Interestingly, the splicing patterns in iPS cells were most similar to those in testis among adult tissues. In addition, the timing of the splicing switches during the reprogramming process varied from gene to gene. These findings indicate that post-transcriptional as well as transcriptional regulation mechanisms are integral parts of the molecular network in the reprogramming process and the pluripotency maintenance. Our results contribute to elucidation of the mechanisms underlying somatic cell reprogramming.

Poster Board Number: F-3306

FUNCTIONAL COMPARATIVE ASSESSMENT OF THE HEMATO-ENDOTHELIAL POTENTIAL OF INDUCED PLURIPOTENT STEM CELLS DERIVED BY VARIOUS APPROACHES.

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Pluripotent stem cell based technologies hold the potential to investigate the basis for or to provide curative therapies for many diseases, including cardiovascular disease, Alzheimer's disease, diabetes and cancers. We have previously used iPS cells derived by reprogramming adult human dermal fibroblasts and using the Yamanaka retroviral protocols, and have demonstrated that specific iPS cell lines contribute not only to the myeloerythroid lineage, but also the B lymphoid lineage (Carpenter et al. 2011; Silk et al. 2011). Additionally, we have shown that particular in-house generated human iPS cell lines can efficiently differentiate towards the cardiac lineage, with sheets of contractile tissue developing (Carpenter et al. 2012), thereby demonstrating their usefulness for high-through-

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put screening of small molecules on different genetic backgrounds. Here, we describe the generation of several new iPS cell lines, and compare the efficiency and differentiation capacity of those generated from human umbilical cord blood erythroblasts and CD34 hematopoietic progenitor cells using OriP plasmid approaches to reprogramming with those iPS lines generated using retroviral strategies. In particular, we have assessed their ability to differentiate towards myeloerythorid, B lymphoid and endothelial lineages. Assessing the impact of different reprogramming technologies and tissue sources on the pluripotency of iPS cells *in vitro*, by functional comparative methods such as assessing B lymphoid differentiation, will compliment the genomic and epigenetic studies that have been traditionally employed. This is also particularly relevant for future applications if we are to identify GMP compliant and cost effective approaches for iPS reprogrammimg, but where subtle aspects of pluripotency requiring more efficient differentiation towards different hematopoietic lineages or towards the vascular lineage are required. Funding: This work is supported by the National Health Service Blood and Transplant (NHSBT) and the National Institute of Health Research (NIHR), UK, under its Programme Grant Scheme (RP-PG-0310-1001, -1003 and -1004). The views expressed in this publication are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health.

Poster Board Number: F-3307

CHEMICAL COMBINATION ENHANCES STEMNESS OF INDUCED PLURIPOTENT STEM CELLS

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Induced pluripotent stem cells (iPSCs) represent a promising unlimited cell source for regenerative personalized medicine. However, the questions about low reprogramming efficiency and late colony formation of the iPSCs were not completely solved yet although it has been reported that the combination of some chemicals improved the reprogramming efficiency of somatic cells into pluripotent cells. So we hypothesized the combination of proper chemicals could improve the reprogramming efficiency as well as the stemness of pluripotent iPSCs. In this study, we generated mouse iPSCs (miPSCs) from the fibroblasts of mouse tail tip in the cell culture conditions with or without chemicals. The miPSCs from both conditions were positive against alkaline phosphatase (AP) and various ES markers such as Oct4, Nanog, Sox2 and SSEA-1, and expressed Oct4, Nanog, Rex-1 and other marker genes. In addition, the miPSCs were differentiated into three germ layers in vitro and formed teratoma in SCID mice *in vivo*. Moreover, the chemical combination improved the reprogramming efficiency of the iPSCs and also induced the early colony formation of the iPSCs: the number of total colony and the AP+ colony in cell culture condition containing chemicals was dramatically increased during iPSCs induction. Furthermore, cell proliferation of the chemical-treated miPSCs, but not chemical-free miPSCs, was very similar to that of J1 mouse ES cells under our cell culture condition. Interestingly, most colonies of the chemical-treated miPSCs on the feeder cells were differentiated when all chemicals were omitted in the culture medium. Therefore, our results show that the use of chemicals could be a valuable tool for the efficient reprogramming and stemness of iPSCs, but the mechanisms should be uncovered.

Poster Board Number: F-3308

COLLAGEN ENHANCES PROLIFERATION OF CARDIAC PROGENITOR CELLS DERIVED FROM INDUCED PLURIPOTENT STEM CELLS VIA ACTIVATING MEK/ERK SIGNALING

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Induced pluripotent stem cells (iPSCs) are a promising alternative source of .patient-specific cardiac progenitor cells (CPCs) and cardiomyocytes for use in drug discovery and regenerative therapies for people with heart diseases. However, their applications are hampered as the generation and expansion of iPSC-derived CPCs and cardiomyocytes are inefficient and vary considerably between iPSC lines. Although several attracted approaches focusing on the manipulation of critical signaling pathways to improve the cardiac differentiation efficiency of iPSCs have been established recently, little is known about the contribution of manipulating extracellular microenvironments to the process of cardiac differentiation from iPSCs. Moreover, the mechanism underlying the renewal and differentiation of CPCs is poorly understood although the CPCs have been identified by multiple markers. Proper methods to efficiently obtain and expand iPSC-derived CPCs *in vitro* remain challenging. To address those issues, we screened sixteen cardiomyocyte inducers on various murine iPSCs and identified that only ascorbic acid (AA) consistently and robustly enhanced the cardiac differentiation in eleven lines including eight without spontaneous cardiac differentiation potential. This cardiac differentiation promoting effect was also observed in human iPSCs. To understand how AA promotes the cardiac differentiation of iPSCs, we did systemic analysis and found that AA took effect at the stage of CPC specification via the specific increase of CPC proliferation but without influence of the apoptotic response and cell viability. The effect of AA on cardiac differentiation could not be mimicked by other antioxidants but was accompanied with significant increases in collagen expressions. Moreover, the AA-promoted CPC proliferation or cardiac differentiation was partially hampered by either knockdown of collagen I or collagen IV expression and abolished by collagen synthesis inhibitors, while the latter effect was partially rescued by adding collagen IV. Further studies showed that AA-enhanced proliferation of CPC population and cardiac differentiation was completely abolished by MEK inhibitor but not JNK, JAK, PI3K or p38MAPK inhibitors, suggesting that the MEK-ERK1/2 pathway is involved in the AA-dependent CPC expansion. This was confirmed by the significantly increased phosphorylation of ERK1/2 in AA-treated cells at differentiation day 5 and this effect was eliminated by the collagen synthesis inhibitors. Our data provide further evidence showing AA is a suitable cardiomyocyte inducer for iPSCs to simply, universally and efficiently improve cardiac differentiation. The findings also highlight the importance of stimulating CPC proliferation by manipulating extracellular microenvironment in guiding cardiac differentiation of the pluripotent stem cells.

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Poster Board Number: F-3309

IMPAIRED EPITHELIAL DIFFERENTIATION OF INDUCED PLURIPOTENT STEM CELLS FROM EEC PATIENTS IS RESCUED BY APR-246/PRIMA-1MET

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Ectodermal dysplasia is a group of congenital syndromes affecting a variety of ectodermal derivatives. Among them, ectrodactyly, ectodermal dysplasia and cleft lip/palate (EEC) syndrome is caused by single point mutation in the p63 gene, which controls epidermal development and homeostasis. Skin defects but also limbal stem cell deficiency are associated with this disease. In this study, we designed a novel cellular model that recapitulated major embryonic defects related to EEC. Fibroblasts from healthy donors and EEC patients carrying two different point mutations in the DNA binding domain of p63 were reprogrammed into induced pluripotent stem cell (iPSC) lines. EEC-iPSC from both patients showed early ectodermal commitment into K18+ cells but failed to further differentiate into K14+ cells (epidermis/limbus) or K3/K12+ cells (corneal epithelium). Treatment with the mutant cells with the small compound APR-246/PRIMA-1Met caused consistent rescue of epidermal and corneal epithelial differentiation, as illustrated by cell morphology and re-activation of specific genes and signaling pathways. This study illustrates the relevance of iPSC for p63 related disorders and may pave the way for future therapy of ectodermal dysplasia syndrome.

Poster Board Number: F-3310

MODELING TRANSITIONS BETWEEN PLURIPOTENT AND PRIMITIVE ENDODERM-LIKE CELLS

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A plethora of events take place transcriptionally and epigenetically during transition of cell fate, such as reprogramming and differentiation. The transcription factors involved in maintaining pluripotency are intricately linked by positive auto-regulatory loop including *Nanog*, *Sox2*, and *Oct4*. Perturbation in any of the three components also perturbs the pluripotent state, leading to the loss of their transcripts and resulting in new differentiated state. As a result, the process of transiting from a pluripotent state to the differentiated states is not reversible except in the case of induced pluripotent stem cells (iPS). In our study with rat embryonic stem cells (ES), we found that cultured rat ES can be guided to become multipotent primitive endoderm (PE) precursor-like cells through medium change; and the process is reversible during a short term cultivation. This system, with its capability of transiting from a pluripotent state to a PE-precursor state and back, is a valuable tool for examining the dynamics of gene expression and provide insights into the regulatory network controlling pluripotency. A Boolean gene regulatory network was constructed to model the transcriptional regulations of the pluripotency markers, *Nanog*, *Sox2*, and *Oct4*, and the primitive endoderm differentiation markers, *Gata6, Gata4,* and *Sox17*. The final model consists of 10 genes and 14 interactions. These interactions were described using Boolean logic operators "AND" when two network components are both required to act on a gene, and "NOT" when the regulation is repressive. We simulated the system starting from all 1024 possible initial conditions and let the system reaches steady states. We showed that the system was able to generate the two stable states corresponding to pluripotent and PE precursor states as observed in culture. An input of LIF and PDGF, simulating a change in culture medium, is sufficient to convert rat ESCs to rat PE precursor-like cells. Over-expression of *Sox2* alone was predicted to be insufficient to convert rat PE-like cells to pluripotent state. Both findings have been verified experimentally. In addition, the model also predicted that knock down of *Gata6* would result in conversion from PE to pluripotent state. Mechanistic model based on differential equations was constructed to evaluate the transitions between the states of pluripotency and PE-precursor. The model incorporates transcriptional process by describing the interactions among transcription factors and their bindings at the regulatory sites. The values of different parameters were estimated using ChIP-seq data from literature. This two pronged approach, using a Boolean network to explore the topology of regulatory network while employing differential equation based model for examining the dynamics of state transitions provide a better strategy for studying the regulation of reprogramming and differentiation toward endoderm lineage.

Pre-clinical and Clinical Applications of Mesenchymal Cells

Poster Board Number: F-3311

MICRORNA 92 MODULATES HYPOXIA INDUCIBLE FACTOR 1- INDUCED SURVIVAL OF HUMAN MESENCHYMAL STEM CELL VIA REPRESSION OF PVHL

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Mesenchymal stem cells (MSCs) have been extensively applied for the restoration of cardiomyocytes loss after myocardial infarction. However the poor viability of MSCs after transplantation and requirement of many cells critically limit the efficacy of this strategy. MicroRNA-92a(miR-92a) is a part of the miR-17-92 cluster, which comprises members with an effect on cell proliferation. Although miR-92a plays an important role in cell survival under hypoxic condition, the role of miR-92a-related survival has yet to be delineated in MSCs. Here, we described the mechanisms of miR-92a for survival and hypoxia inducible factor-1α (HIF-1α) stabilization in MSCs. MiR-92a mimic induced expression of HIF-1α and miR-92a in MSCs, respectively, which was confirmed by western blot and real time-PCR analysis. Overexpression of miR-92a regulated expression of apoptosis-related factors such as p-AKT, p-ERK, Bcl-2, Bax, annexin V/PI in hypoxic condition. In addition, increased survival was detected in hypoxic condition by MTT assay. MiR-92a was a possible target of pVHL, which identified through assessed activity luciferase reporter construct containing the 3' UTR of pVHL and expression of pVHL. These data suggested that miR-92a plays a key role in survival of MSCs under hypoxic condition.

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Poster Board Number: F-3312

MICRORNA 371-373 CLUSTER PROMOTES THE SURVIVAL OF HUMAN MESENCHYMAL STEM CELLS

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Mesenchymal stem cells (MSCs) showed self-renewal and multi-pluripotent characters and possibilities for tissue engineering and regeneration, but some problems such as pluripotency of isolated MSCs and sufficient amounts of cells for therapy remain unsolved. MicroRNAs (miRNAs) are small non-coding RNA and play important roles in stem cell differentiation and self-renewals. To address self-renewal maintenance by miR-371-373 cluster, miR-371-373 cluster was primarily transfected into MSCs. The cells showed higher self-renewal MSCs in serum free condition, based on upregulated expression of DNMT1, MLL5, Kdm2b and Sirt1 genes, and protected from serum starvation-induced apoptosis through TUNEL assay. We further confirmed that miR-371-373 inhibited differentiation to other cell types, based on downregulated expression of FZD1, GDF9, IL6R and BMP5, which genes are involved in differentiation. Also, miR-371-373 influenced the cell cycles; the quiescent G0/G1 phase cells are more than S+G2/M phase (active proliferation). Taken together, we suggested that miR-371-373 cluster may be involved in maintainence the undifferentiated state and self-renewal property of MSCs.

Poster Board Number: F-3313

THE USE OF AUTOMATED IMAGE ANALYSIS AND HIGH CONTENT SCREENING TO DISTINGUISH LATE PASSAGE MESENCHYMAL STEM CELLS FROM OSTEOSARCOMA CELLS

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Introduction: MSCs are attractive candidates for autologous cell therapies in regenerative medicine. Their application involves culture expansion of isolated MSCs, especially as we scale up to the treatment of human disease. Several reports have suggested that extended MSC culture is associated with chromosal abnormality and potential malignant transformation. MSCs are also considered the likely cancer stem cell for some bone tumours. Here, we have examined whether late passage culture expanded MSCs can be distinguished from transformed cells using routine microscopy, computerised live cell imaging/analysis, and high content screening (HCS) techniques. *Methods:* Human MSCs and SAOS2 osteosarcoma cells were established as single cell type and co-cultures, where co-cultured cells were fluorescently tagged to confirm their identity. Cultures were monitored by continuous live cell imaging and analysis using the Cell IQ platform and via HCS of fixed cultures for stem cell, proliferation and tumour markers, as well as cell cycle analysis. *Results*: Based on pattern recognition of digitised phase contrast images, the Cell IQ platform identified: (i) 65% ± 6% of cells as MSCs versus 15% ± 4% of cells as SAOS2 , in MSC monocultures; (ii) 65% ± 2% of cells as SAOS2 versus 8% ± 2% of cells as MSC, in SAOS2 monocultures; (iii) 54% ± 5% of cells as MSC versus 32% ± 5% of cells as SAOS2, in 50:50 co-cultures. Significant differences in quantitated cell positivity for several markers were detected using HCS in MSCs vs SAOS2 fixed cultures, including alkaline phosphatase, Ki-67 antigen and Oct3/4, whereas no difference was detected in phospho-retinoblastoma immunoreactivity. The morphology of the MSCs markedly differed to that of SAOS2 cells, which was determined quantitatively via HCS after beta-tubulin immunolocalisation. There was also a greater prevalence in MSC cultures for senescence associated β galactosidase-positive cells. *Discussion*: We have demonstrated that computerised live cell image analysis and HCS techniques can help identify whether MSC cultures may be contaminated with tumour cells. The cell phenotypes established using these methods will help clinical programmes develop release criteria for MSC therapies to ensure the safety of using culture expanded MSC. Such detailed and relatively rapid profiling will also enhance our understanding of MSC phenotype, which even as clonal populations are considered highly heterogeneous in nature.

Poster Board Number: F-3314

HUMAN MESENCHYMAL STEM CELLS STIMULATE EAHY926 ENDOTHELIAL CELL ADHESION AND MIGRATION: A COMBINED PROTEOMIC AND *IN VITRO* ANALYSIS OF THE INFLUENCE OF DONOR-DONOR VARIABILITY AND SECRETED EXTRACELLULAR MATRIX PROTEINS.

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Mesenchymal stem cells (MSCs) can stimulate angiogenesis within a wound environment and evidence suggests that this effect is likely mediated by paracrine interactions with the endogenous cells present. Here we report that MSC conditioned medium (MSC-CM) from 3 different donors stimulated EaHy-926 endothelial cell adhesion and cell migration, using the scratch assay. This stimulatory effect was donor-dependent. MALDI-TOF/TOF mass spectrometry demonstrated that whilst type I collagen and fibronectin was secreted by all of the MSC cultures, decorin was secreted only by the MSC culture that was least effective in stimulating EaHy-926 cells. When these individual extracellular matrix components were then tested as culture substrata, EaHy-926 cell adherence was greatest upon culture plates coated with fibronectin and least adherent on plates coated with decorin, with type I collagen coated plates intermediate. Similarly, EaHy926 cell migration and scratch-wound closure was quickest on fibronectin-coated plates and slowest on decorin-coated plates, with type I collagen-coated plates intermediate. However, on each of these substrata scratch-wound closure was significantly increased in the presence of MSC-CM. These data suggest that of those MSC-secreted factors that constitute extracellular matrix proteins, fibronectin is likely to be pro-angiogenic whilst decorin may be considered anti-angiogenic. They also illustrate a need to screen for donor-donor variability in the secretome of MSCs, as this will both increase our understanding of their wound healing activity and also inform their appropriate clinical application.

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Poster Board Number: F-3315

EARLY DYNAMIC CHANGE OF GCSF AND INTERLEUKIN 1-BETA EXPRESSION AFTER AUTOLOGOUS BONE MARROW CELL INFUSION ARE IMPORTANT TO START THE REPAIR OF CIRRHOSIS LIVER

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Obejective: We developed autologous bone marrow cell infusion (ABM*i*) therapy for liver cirrhosis patients (Stem Cells 2006). The effectiveness and safety for liver cirrhosis (LC) patients was shown following clinical study. In this study, we tried to analyze the early change of cytokine expression in serum after ABM*i* therapy. We analyzed chronological changes in 19 serum cytokines as well as levels of specific cytokines between in patients after ABM*i* therapy and in a mouse model of cirrhosis generated using green fluorescent protein (GFP)/carbon tetrachloride (CCl_4) (JB 2003). Method: We measured expression profiles of cytokines in serum samples collected from 13 LC patients before and at 1 day and 1 week after ABM*i* therapy using Bio Plex suspention array system (BIO RAD company). We measured interleukin(IL)-1 α, IL-1 β, IL-2, IL-3, IL-4, IL-5, IL-6,IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-17, Eotaxin, G-CSF, GM-CSF, IFN-γ, KC, MCP-1, MIP-1 α, MIP- β, RANTES, TNF-α, basic FGF and VEGF. Child-Pugh scores significantly improved in all of these patients after 6 month after ABM*i* therapy . To analyze the meaning of early cytokine change, we compared chronological changes in serum cytokine expression in humans and in the model mice at 1 day and 1 week after BMC infusion. We isolated GFP positive BMC from GFP Tg mice and infused $1x10^5$ BMC via tail vein to CCl4 induced serial liver cirrhosis mice. After BMC infusion we continued liver damage by CCl4 injection (GFP/CCl4 model, JB 2003). . We got serum from control CCl4 treated mice and BMC infusion group at 1day and 1 week. Next, we used Bio Plex suspention array system to quantify cytokine erxpression. Result: Among 19 cytokine, both G-CSF and IL-1β in serum was found to show same chronological change pattern between human and mice model. Serum G-CSF and IL-1β was decreased after BMC infsuion at 1day and increased at 1 week. Next we examined changes in cytokine expression in cirrhosis liver before, at 1, 2, 3 and 4 weeks after BMC infusion using GFP/CCl4 mice model. Both G-CSFand IL-1β were undetectable in the liver tissues before and at 1 week after BMC infusion, but increased at 2 weeks and continued until 4 weeks after infusion. The BMC infusion induced an early decrease of both G-CSF and IL-1β in serum and an increase in the cirrhosis liver in GFP/CCl4 model. Conclusion: These dynamic early changes of G-CSF and IL-1β after autologous BMC infusion might be important to start for repair of liver cirrhosis after BMC infusion.

Poster Board Number: F-3316

MSC DERIVED FROM HUMAN BONE MARROW INFUSION THERAPY WILL BE A NEW CELL THERAPY FOR LIVER CIRRHOSIS

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[Background and objectives] We developed "autologous bone marrow cell infusion (ABM*i*) therapy" for human decompensated liver cirrhosis (LC) patients (Stem Cells 2006). The efficacy and safety of this treatment was shown in multicenter clinical studies. The basic concept of ABMi therapy was based on the finding that bone marrow cell (BMC) infusion improved liver fibrosis in cirrhosis liver and activated resident liver progenitor cell and hepatocyte and improved liver function. In this study we tried to develop a new cell therapy using cultured bone marrow cell to cure more severe LC patients because ABM*i* therapy is difficult for LC patient who can not get general anesthesis to obtain bone marrow. [Methods] We developed a cirrhosis model mice by repeatedly administering carbon tetrachloride to NOD-SCID mice for 6 weeks. Human bone marrow mononuclear cells (Lonza, 2M-125A) were seeded in culture flasks and cultured in 10% FBS-DMEM. Cultured BMC was differentiated into Mesechymal stem cell during 20 days. The characteristics of the cell fractions of culturing BMC were evaluated by FACS. 5×10^5 human BM-derived MSC were infused into the CCl4 induced liver cirrhosis NOD-SCID mice via the caudal vein. 4 weeks after MSC infusion, liver fibrosis was assessed by sirius-red staining. Activated stellate cell was also analyzed by αSMA staining. The expression of MMP9 and TIMP1 expression in cirrhosis liver was analyzed by RT-PCR. [Results] 20 days culturing cells derived from human bone marrow was differentiated into mesenchymal stem cells (CD73/CD90/CD105 positive and CD45 negative) and the number of cells increased 43-fold. We also confirmed multiple cell lineage of this MSC. Cultures MSC infusion significantly inhibited liver fibrosis ($p<0.05$) in LC NOD-SCID mice. In this condition, MMP9 expression was significantly increased with decrease of TIMP1 expression. In this condition,αSMA positive stellate cell was decreased. [Conclusion] We demonstrated that cultured MSCs derived from human BM would be useful to improve liver fibrosis and that MSCs in the cultured cells would proliferate sufficiently to use for cell therapy. We believe that the infusion of cultured human BM-derived cells might improve liver fibrosis by MMP9 activation with inhibition of collagen production by stellate cells. Culturing MSC infusion derived from human bone marrow will be a new cell therapy for liver cirrhosis patients.

Poster Board Number: F-3317

HUMAN FETAL MESENCHYMAL STEM CELLS AND ENDOTHELIAL PROGENITOR CELLS FOR THE GENERATION OF ENGINEERED BONE GRAFTS: A PRE-CLINICAL STUDY

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Aim: Non-union fractures represent a significant clinical burden, and cannot be treated with existing modalities, creating an urgent

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need for alternative approaches. Over the past decade, tissue engineered bone grafts (TEBG) have emerged as a potential therapeutic option for the treatment of such defects. We have previously described the generation of bone grafts through an integrative approach combining the use of human fetal mesenchymal stem cells (fMSC), endothelial progenitor cells (EPC) and resorbable scaffolds primed in a bio-mechano-stimulatory bioreactor. These bone grafts have been shown to be highly osteogenic in nature and efficacious in repairing segmental non-union fracture defects in rodents. We extend our studies here to a pre-clinical large animal model in a minipig tibial segmental defect model. Methods: Two experimental groups were implemented in this study to evaluate the safety and relative efficacy of TEBG generated using (i) fMSC (n=10) or (ii) a combination of fMSC and EPC (n=6), versus acellular scaffolds as a control arm. Polycaprolactone-Tri Calcium Phosphate (PCL-TCP) scaffolds were loaded with 4 million fMSC or in combination with 4 million EPC, and subsequently cultured in a biaxially rotating bioreactor for seven days in bone-inductive medium. Engineered bone grafts were then retrieved and rinsed with saline prior to implantation. Samples were also retrieved for assessment of cell viability, mineralisation and septicity. A minipig model of segmental tibial defect was developed for this study. Briefly, an 18mm segmental defect was introduced into the tibia, into which the TEBG was placed and subsequently stabilised by internal fixation. Post-operative monitoring comprised of serial X-rays and Computed Tomography (CT) scans. Results and Discussions: Large TEBG were successfully generated, with good viability and even distribution through the cross-section of the grafts. In addition, EPC were found persist after seven days of culture in bone-inductive medium. EPC-MSC group was also found to exhibit increased mineral content, suggesting a role for EPC in exerting pro-osteogenic influences in the generation of TEBG. Septicity tests confirmed the absence of microbial contamination and asepsis required for implantation. All animals survived the operation. Post-operative scans demonstrate the implant sites to be stable at three months. No lesions or other evidence for tumorigenic activity were observed in all animals. Conclusions: A relevant large animal model crucial for clinical translation was developed to evaluate TEBGs. Protocols were developed for the monitoring and long-term follow-up studies on implanted pigs. A total of 16 pigs were implanted, with neither death nor evidence of tumorigenic activity or osteosarcommas, suggesting that the delivered cells fall within the tolerated dose. Further work is underway to elucidate cell fate and efficacy of the approach.

Poster Board Number: F-3318

COMBINATION OF HUMAN EYELID ADIPOSE STEM CELLS IMPLANTATION AND 17-BETA-ESTRADIOL ADMINISTRATION IMPROVE SPINAL CORD INJURY REPAIR

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Stem cell transplantation and pharmaceutical administration represent two promising strategies for the repair of spinal cord injury (SCI). However, the synergistic effect of the combination of these two approaches has yet to be evaluated. Here, we report that 17-β-estradiol (E2) administration together with human eyelid adipose-derived stem cells (hEASCs) transplantation synergistically improved the functional repair of SCI. Our results showed that E2 boosted the survival of grafted hEASCs by reducing apoptosis through regulating the expression of caspase-3 and bcl-2. E2 also increased the secretion of growth factors by hEASCs. The survived hEASCs preferentially differentiated into neurons instead of astrocyte. Furthermore, E2 combined with hEASCs transplantation synergistically reduced cavity formation and promoted axon remyelination as well as motor function recovery. Our study demonstrated that combination of estrogen administration and hEASCs transplantation improved functional recovery of SCI. It provides a promising chemo-cell cocktail strategy for the treatment of incurable neurodegenerative diseases.

Poster Board Number: F-3319

PERIURETHRAL INJECTION OF AUTOLOGOUS ADIPOSE-DERIVED STEM CELLS FOR THE TREATMENT OF INTRACTABLE STRESS URINARY INCONTINENCE IN 5 PATIENTS UNDERGOING PROSTATIC SURGERY: CLINICAL NOVEL FRESH AND UNCULTURED CELL THERAPY FOR UROLOGY

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Stress urinary incontinence can have a significant impact on a patient's quality of life, resulting in involuntary release of urinedue a weakened urethral sphincter. The condition is more common in women and often comes about following child birth or menopause. It is estimated that approximately 9 million people in Japan, and more than 13 million women in the U.S., are affected by stress urinary incontinence. Objectives: To report a novel cell therapy using freshly isolated, uncultured and autologous adipose tissue-derived stem cells (ADSC) for stress urinary incontinence caused by urethral sphincteric deficiency and the outcomes in five cases undergoing periurethral injection of stem cells for the treatment of urinary incontinence more than persistent two year after radical prostatectomy(n=4) and transurethral resection of prostate(n=1). Methods: Two patients with moderate stress incontinence after radical prostatectomy were enrolled. After liposuction of 200-250 mL of adipose tissue from the abdomen, we isolated ADSC from this tissue by using the Celution system. Subsequently, the isolated ADSC and a mixture of stem cells and adipose tissue were transurethrally injected into the rhabdosphincter and submucosal space of the urethra, respectively. Short-term outcomes during a 24-48 week follow-up were assessed by a 24-h pad test, a validated patient questionnaire, urethral pressure profile, transrectal ultrasonography, and magnetic resonance imaging. Results: Urinary incontinence progressively improved after 2-4 weeks of injection up to 12 weeks in terms of decreased leakage volume in a 24-h pad test, decreased frequency and amount of incontinence, and improved quality of life as per the questionnaire. In urethral pressure profile, both maximum urethral closing pressure and functional profile length increased. Ultrasonography and magnetic resonance imaging showed sustained presence of the injected adipose tissue. Enhanced ultrasonography showed a progressive increase in the blood flow to the injected area. No significant adverse events were observed peri- and postoperatively. Conclusion: This preliminary study showed that periurethral injection of the autologous ADSC is a safe and feasible treatment modality for stress urinary incontinence.

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Poster Board Number: F-3320

PANCREATIC LINEAGE DIFFERENTIATION OF HUMAN DENTAL PULP CELL

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Objectives:The need of the regenerative medicine of the pancreas tissues would greatly increase in near future, since their four types of the cells involve each specific function. However, it has not been reported that a protocol produces a large numbers of highly purified pancreatic differentiated cells from somatic stem cells. The ability of dental pulp stem cells to differentiate towards endodermal lineages was recently demonstrated, such as hepatic cells. We previously established a novel culture protocol for pulp stem cells which can produce enough number of cells for transplantation to humans. The present study is to assess multilineage potential for differentiation of dental pulp stem cells, specifically towards *pancreatic lineage* in serum-free condition Materials and Methods: Cell cultures were isolated from deciduous tooth and third molar pulp and were grown in DMEM supplemented with 10 % FBS. After 3 to 5 passages $CD117^{+}$cells were magnetically separated. Cells were characterized for a panel of stem cell markers by immunocytochemistry and flow-cytometry. Cells between 3-5 passages were subjected to the following differentiation protocols: - For adipogenic differentiation the cells were grown in DMED supplemented with 1μM Dexamethasone, 50 μM Indomethacine, 1% ITS-x and 100 μM ETF. - For osteogenic differentiation the medium was DMEM plus 10 μM Dexamethasone, 100μM Ascorbic acid, 10mM β-glycerophosphate, 1% ITS-x and 100 μM ETF. -For endodermal commitment 2% FBS, 20 ng/ml hepatic growth factor (HGF) and 10 ng/ml acidic fibroblast growth factor (aFGF) were added to DMEM for 5 days. For pancreatic commitment the medium was changed with serum-free αMEM supplemented with 20 ng/ml HGF, 10 ng/ml FGF, 20 ng/ml EGF, 100 μM β-mercaptoethanol and 100 μg/ml ETF, for 7 days. For pancreatic maturation medium was changed with serum-free IMDM supplemented with 20 ng/ml HGF, 100 μg/ml ETF and 10mM nicotinamide for another 7 days. Results: Both mesenchymal cell lines from DTPC and WTPC were proven to be positive for a panel of pluripotent stem cell markers: CD117, nanog, nestin, CD44H, alkaline phosphatase, oct 3/4, citokeratin 19, osteonectin and p63. After pancreatic differentition, insulin, glucagon, pancreatic polypeptide and somtostatin (red - D) were found positive (n=5) with indirect immunostaining. After the differentiation the percentage of DTPSC cells expressing endocrine markers insulin, glucagon, somatostatin and pancreatic polypeptide, and the exocrine marker pancreatic amylase-2 was found by flow-cytometry. Conclusion: The present results demonstrate the ability of $CD117^{+}$ dental pulp stem cell cultures to differentiate to ectodermal, mesodermal and endodermal type of cells in serum-free conditions. These cells also acquired characteristics of pancreatic endocrine and exocrine cells. Dental pulp mesenchymal cells may therefore have great potential for future cell therapy of diabetic patients.

Poster Board Number: F-3321

ENHANCED REGENERATIVE POTENTIAL OF STEM CELLS ISOLATED FROM INFLAMED DENTAL PULP TISSUE OF HUMAN FUNCTIONAL DECIDUOUS TEETH BY FIBROBLASTIC GROWTH FACTOR 2 APPLICATION

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Background: Stem cells can be isolated from inflamed dental tissuesincluding dental pulp and periodontal ligaments, however, their reduced regenerative potential has limited the clinical applications. In the present study, we successfully isolated mesenchymal stem cells (MSCs) from inflamed dental pulp tissue of human deciduous teeth and demonstrated that regenerative potential could be enhanced by fibroblastic growth factor-2 (FGF-2) treatment during *ex vivo*expansion. Materials and methods: Stem cells were obtained from inflamed dental pulp tissue of human functional deciduous teeth (iSHFD) during root canal treatments. Characterization of the isolated stem cells expanded in FGF-2 were conducted using colony forming assay, proliferation, migration, differentiation, and in vivoectopic transplantation. Also self-renewal potency was demonstrated by in vivoretransplantation. The effects of FGF-2 (iSHFD/FGF-2) were evaluated and compared to stem cells from iSHFD. Results: MSCs can be isolated from inflamed pulp tissue of functional deciduous teethand characterized as consisting potential qualities of MSCs similar to stem cells from human exfoliated deciduous teeth (SHED). For FGF-2 application assessment, we confirmed that FGF-2 treatment to iSHFD during expansion enhanced colony forming efficiency. FGF-2 application also increased proliferation and migration potential significantly while decrease differentiation potential in vitro. However, when iSHFD/FGF-2 was ectopically transplanted in vivo, increased dentin-like structure formation was observed.As an underlying mechanism for its enhanced regenerative characteristics, we found that self-renewal potential also increased and more mineral formation occurred. Conclusion: The results of our study confirmed that stem cells from inflamed pulp tissues of human deciduous teeth can be a good source for future clinical application with FGF-2 expansion. Such approach may provide a novel way to use discarded inflamed tissues in clinical regenerative therapy. Acknowledgement: This study was supported by Mid-career Researcher Program through NRF grant funded by the MEST (No. 2009-0078884), and a grant of the Korea Health technology R&D Project, Ministry of Health & Welfare, Republic of Korea. (A100443).

Poster Board Number: F-3322

MULTIFACETED NERO-REGENERATIVE ACTIVITIES OF HUMAN DENTAL STEM CELLS PROMOTE LOCOMOTOR RECOVERY AFTER RAT SPINAL CORD INJURY

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Spinal cord injury (SCI) often leads to persistent functional deficits due to severe neuron and glial loss, and to limited axonal regeneration after injury. During the acute phase, the focal mechanical insult disrupts tissue homeostasis. This triggers secondary injury pro-

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cesses, in which multiple destructive cascades cause the necrotic and apoptotic death of neurons, astrocytes, and oligodendrocytes that spreads beyond the initial injury site and leads to irreversible axonal damage and demyelination. Subsequently, reactive astrocytes and oligodendrocytes near the site of injured spinal cord (SC) respectively produce chondroitin sulfate proteoglycans (CSPG) and myelin proteins (including myelin-associated glycoprotein/MAG, Nogo, OMG, Netrin, Semaphorin, and Ephrin). These extracellular molecules function as axon growth inhibitors (AGIs), acting through the intracellular Rho GTPase signaling cascade. These multiple pathogenic signals synergistically accelerate the progressive deterioration after SCI. Therefore therapeutic strategies for functional recovery from SCI must exert multifaceted reparative effects against a variety of pathogeneses. We report here the remarkable neuro-regenerative activity of tooth-derived stem cells, for the functional recovery of SCI. Our study revealed that engrafted tooth-derived stem cells exhibited three major therapeutic benefits for recovery after SCI, including (1) inhibition of the SCI-induced apoptosis of neurons, astrocytes, and oligodendrocytes, which promoted the preservation of neural fibers and myelin sheaths, (2) regeneration of the transected axon through the direct inhibition of multiple AGI signals by paracrine mechanisms, and (3) replacement of lost or damaged oligodendrocytes after SCI through specific differentiation into mature oligodendrocytes under the extreme conditions of SCI. To our knowledge, the neuro-regenerative activities (2) and (3) are unique to tooth-derived stem cells, and are not exhibited by any other previously described stem cells. Thus, our data demonstrate that tooth-derived stem cells may provide significant therapeutic benefits for treating the acute phase of SCI through both cell-autonomous and paracrine/trophic regenerative activities.

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PRECONDITIONING HUMAN MESENCHYMAL STEM CELLS WITH INTERFERON-GAMMA IMPROVES GRAFT VERSUS HOST DISEASE

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Background: It is important to overcome the limitations such as graft rejection and graft versus host disease (GVHD) in allogeneic hematopoietic stem cell transplantation. Mesenchymal stem cells (MSCs), which evoke only minimal immune reactivity, may have anti-inflammatory and immunomodulatory effects. Purpose: In this study, we aimed to identify the immunomodulatory properties of human naive MSCs and interferon (IFN)-γ-treated MSCs and elucidate the possible mechanism of their properties for clinical treatment of allogeneic conflicts using MSCs. We have also tried to comparative analysis about the immunomodulatory properties of MSCs derived from adult human tissues, including bone marrow (BM), adipose tissues (AT), umbilical cord blood (CB), and cord Wharton's jelly (WJ), in vitro and *in vivo* models. Results: AT-MSCs, CB-MSCs, and WJ-MSCs suppressed phytohemagglutinin (PHA)-induced T-cell proliferation as effectively as did BM-MSCs. Levels of IFN-γ secreted from activated T-cells increased over time, but these levels were significantly reduced when co-cultured with each type of MSCs. In addition, expression of indoleamine 2,3-dioxygenase (IDO) increased in MSCs treated with IFN-γ via JAK/STAT1 signaling pathways. Treatment with anti-IFN-γ antibodies, JAK inhibitor, STAT1 siRNA or IDO shRNA restored PHA-induced T-cell proliferation. An antagonist, 1-methyl-L-tryptophan, also restored PHA-induced T-cell proliferation, suggesting that IDO contributes to IFN-γ-induced immunosuppression in MSCs. Moreover, infusion of IFN-γ-treated MSCs reduced symptoms of human peripheral blood-derived mononuclear cells-induced GVHD in NOD/SCID mice, which resulted in an increase of survival rate of *in vivo* GVHD model compared with naive MSCs-infused mice, but not with IFN-γ-treated IDO down-regulated MSCs. Conclusions: These data indicate that IFN-γ produced by activated T-cells is correlated with induction of IDO expression in MSCs by IFN-γ receptor/JAK/STAT1 pathway, which results in suppression of T-cell proliferation. Our findings also suggest that IFN-γ pre-conditioned-MSCs, which derived from BM, AT, CB, or WJ, could be used for clinical treatment of allogeneic conflicts.

Poster Board Number: F-3324

HUMAN PLACENTA-DERIVED MULTIPOTENT CELLS (PDMCS) MODULATE CARDIAC INJURY BY PROMOTING ANGIOGENESIS THROUGH SECRETED PARACRINE FACTORS

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Recent studies have shown that human placenta-derived multipotent cells (hPDMCs) are capable of multilineage differentiation. In addition, the lack of ethical concerns in procurement of these multilineage progenitors and their immunomodulatory properties make them good candidates for cell therapy of damaged organs. We hypothesized that hPDMCs might have the capacity for cardiac repair. In this study, we show that transplantation of hPDMCs into the hearts of severe combined immunodeficient (SCID) mice after left anterior coronary artery (LAD) ligation significantly improves left ventricular function as analyzed by ultrasonographic follow-up, with significantly enhanced vascularity in the cell-treated group. The proangiogenic effects were further confirmed *in vitro* by the enhancement of endothelial cell tube formation with conditioned medium (CM) from hPDMCs compared to CM from bone marrow mesenchymal stem cells. These proangiogenic effects were mediated by hPDMC secretion of various pro-angiogenic factors including HGF and IL-8. Our findings offer mechanistic insights into a promising therapeutic strategy using hPDMCs to treat severe cardiovascular diseases.

Poster Board Number: F-3325

IN VIVO THERAPEUTIC EFFECTS OF CONDITIONED MEDIUM FROM HUMAN ADIPOSE TISSUE-DERIVED STEM CELLS ON RAT STROKE MODEL

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Stem cell therapy is a promising approach for stroke. However, low survival rates and potential tumorigenicity of implanted cells could undermine the efficacy of the cell-based treatment. The use of stem cell-conditioned media (CM) may be a feasible approach to overcome the limitations. Especially, specific stem cell culture con-

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dition and continuous infusion of CM into ischemic brains would have better therapeutic results. The CM was prepared by culturing human adipose-derived stem cells in a three-dimensional spheroid form to increase the secretion of angiogenic/neuroprotective factors. Ischemic stroke was induced by standard middle cerebral artery occlusion methods in the brain of 8 week-old Sprague-Dawley rats. Continuous infusion of CM or αMEM media (0.5 μl/h) into the lateral ventricle was initiated 8 days after the surgery, and maintained for 7 days. Alteration in the motor function was monitored by the Rota rod test. Infarction volume and the number of microvessels or TUNEL-positive neural cells were analyzed 15 days after the surgery. Compared with αMEM media, continuous CM infusion reduced the infarction volume and maintained motor function. The number of CD31-positive microvessels and TUNEL-positive neural cells significantly increased and decreased, respectively, in the penumbra regions. Although the apoptosis of all neural cell types decreased, reduction in the microglial apoptosis and astrogliosis was prominent and significant. In this study, the therapeutic effects of the CM against stroke were confirmed in an animal model. Increased endothelial cell proliferation, reduced neural cell apoptosis, and milder astrogliosis may play important roles in the treatment effects of CM.

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IMMUNE PROPERTIES OF HUMAN CD47+ SUB-POPULATION FROM PLACENTA-DERIVED MESENCHYMAL STEM CELLS

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Background: Mesenehymal stem cells(MSCs)modulate the immune function of the major immune cell populations involved in alloantigen recognition and elimination, including antigen presenting cells, T cells, B cells and natural killer cells. MSCs can be clinically applied for graft-versus-host and autoimmune diseases. The placenta has been suggested as an abundant, ethically acceptable, less immunogenic and easily accessible source of MSCs called PMSCs. Here we aimed to identify and isolate a sub-population of PMSCs with high positive surface antigen about CD47. Objective: To isolation and study the immune properties of human CD47 positive Sub-population of PMSCs *in vitro*. Methods and Results: The PMSCs were isolated and cultured in the normal procedure. Flow cytometry sorting was used to isolate the CD47 positive sub-population of PMSCs. The phenotype analysis showed that the cell surface marker CD29, CD44, CD73, CD90, CD105 was positive, while CD14, CD34, CD45 and HLA-DR was negative in CD47 positive sub-population cells. Differentiation of CD47 positive sub-population towards adipogenic, osteogenic and neurogenic lineages was performed *in vitro*. The results indicate that CD47+ sub-population could be differentiated into not only mesenchymal, but also ectodermal lineage. The mixed lymphyocyte proliferation assays were using standard methods. were acquired from peripheral blood by density gradient centrifugation.The CD47+ sub-population from PMSCs and activated Mononuclear cells were co-cultured.The proliferation of lymphocytes was measured by MTT method; The expression of the immunoreactions factors IFN-γ, TNF-α, and TGF-β were evaluated by ELISA. Results The PMSCs CD47 positive sub-population could not induce the proliferation of lymphocytes. The proliferation of lymphocytes could be inhibitad to different extents by adding different ratios of CD47 positive sub-population.Meanwhile, The PMSCs CD47 positive sub-population could reduce immunoreactions factors in the mixed lymphocyte reaction experiment. Conclusions Compared with PMSCs, The PMSCs CD47 positive sub-population has more immunoregulated capability. *Correspondence: WEI Jun E-mail: lydiajunwei@hotmail.com (The research is supported by the National Natural Science Foundation of China, NO.30960176)

Poster Board Number: F-3327

RIC-8 REGULATES G PROTEINS FUNCTION OF HUMAN MESENCHYMAL STEM CELLS FOR MULTIPLURIPOTENT CHARACTERS

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Guanine nucleotide-binding proteins(G proteins) are signal transducers that communicate signals from many hormones, neurotransmitters, chemokines, autocrines and paracrine factors. Ric-8(Resistance to inhibitors of cholinesterase 8)acts as a guanine nucleotide exchange factor (GEF) for G protein α-subunit (Gα) and switches different heterotrimeric G proteins which regulate of virtually all cellular functions via G protein-coupled receptors (GPCRs). However, the functions of Ric-8in the survival of bone marrow-derived human mesenchymal stem cells (hMSCs) are unknown.In this study, we used a Ric-8 siRNAt o examine the roles of Ric-8 in survival of hMSCs.The suppression of Ric-8 by siRNA reduced the membrane association of $G\alpha_q$, and also diminished the ERK activation which is mediated by $G\alpha_q$-coupled receptor in hMSCs. In addition, Ric-8 regulated expression of apoptosis-related proteins (Bcl-2, Bax,Caspase-3, Caspase-8, and Cytochrome-c). The knock down of Ric-8 influenced downstream effector proteins including Rho, Ras, and Rac. Taken together, these data indicate that Ric-8 has crucial roles in a variety of cellular function such as cell cycle, cell proliferation, and other common cellular functions for hMSC survival.

Poster Board Number: F-3328

SUCCESFUL TREATMENT OF ALLOGRAFT REJECTION AFTER RENAL TRANSPLANTATION WITH HUMAN AUTOLOGOUS BONE MARROW DERIVED MESENCHYMAL STROMAL CELLS

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Despite excellent short-term results, the long-term survival of transplanted kidneys has not improved accordingly, contributing to the shortage of donor organs. While allo-immunity and side effects of immune suppressive drugs have been identified as the main drivers of early interstitial fibrosis and tubular atrophy (IFTA) no effective treatment options have emerged. Mesenchymal stromal cells (MSCs) have anti-inflammatory and anti-fibrotic properties and reverse renal injury in preclinical models. We report initial results of 5 kidney allograft recipients who received expanded autologous

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bone marrow derived MSCs (106 cells/kg twice iv, 7 days apart) because of rejection and/or an increase of IFTA in the protocol biopsy (Clinical trials NCT00734396). MSCs fulfilled the release criteria and demonstrated immunosuppressive capacities comparable to MSCs from healthy controls *in vitro*. The MSC infusion was well tolerated and there were no adverse events. Interestingly, all patients that received MSCs demonstrated a profound reduction in proliferation of patient PBMC 12 weeks after MSC infusion upon stimulation with donor specific PBMCs, while the response to third party PBMCs was more variable. In 2 patients we had clinical indications to do a third biopsy and are able to report for the first time on the potential effects of MSC infusion in allograft rejection. Both of the patients received a kidney 2 HLA-DR mismatched living donor. In patient 1 the biopsy at 6 months showed T cell mediated rejection with severe tubulitis (Banff 1b). In patient 2 the 6 month biopsy showed multiple foci of tubulitis and mild IFTA, indicating a borderline rejection (Banff<1a). MSCs were infused while immunosuppressive drugs remained unchanged. A third biopsy was taken for clinical reasons (patient 1 because of severity of rejection in protocol biopsy; patient 2 because of a transient creatinine rise, which could later be related to drug-induced hypercalciemia). In both patients the infiltrate had disappeared and there were no signs of IFTA after the MSC infusion. Although maintenance immune suppression remained unaltered, three patients developed an opportunistic viral infection. Patient 3 developed a BK-virus associated nephropathy 21 weeks after the infusion. Serum creatinine returned to normal (from 140 to 104 µmol/l) with reduction of immune suppressive dose. In patient 4 a late primary CMV infection (more than 6 months after prophylactic valganciclovir discontinuation) was diagnosed 2 weeks after MSC infusion. Also this patient recovered uneventful with antiviral therapy and reduction of immune suppression. In patient 5 a low grade CMV viral load persisted in the months after MSC infusion, despite reduction of clinical immune suppression. In conclusion, these first clinical observations support the potential of MSCs as novel cell therapy to prevent acute renal allograft rejection and further option to reduce exposure to (nephro)toxic drugs. The observed systemic immune suppression after MSC infusion implies careful monitoring of opportunistic virus infections.

Poster Board Number: F-3329

STUDY THE SIDE EFFECTS OF INJECTION OF AUTOLOGOUS MESENCHYMAL STEM CELLS IN PATIENTS WITH HIP OSTEOARTHRITIS

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Objective: The purpose of this study was to evaluate the safety of single dose intra-articular injection of autologous expanded mesenchymal stem cells in patients with severe hip osteoarthritis candidate for total hip replacement. Method: After obataining approval of local medical ethics committee and informed written consent form, patients with clinical and radiological evidence of hip osteoarthritis were recruited. MRI of the affected hip was obtained preoperatively and 6 months after treatment in the saggital, coronal and axial planes. Autologous bone marrow mesenchymal stem cells were obtained from 80-100 ml bone marrow aspiration. Minimum 2×107 cells were then applied to the subject`s hip under the guide of fluoroscopy through an inpatient procedure. Patients were assessed clinically with scoring system (HHS, VAS, WOMAC) preoperatively as well as 2 and 6 months postoperative to measure pain reduction and joint function improvement. Results: Six patients with the mean age of 41.5±7.63 years (f=2, M=4) entered the study. Two patients did not attend to one of the follow up visits. During 6 months after implantation, no local or systemic adverse event was observed. Clinical improvement evaluated by VAS, WOMAC and HHS six months post injection. Also MR imaging illustrated resurfacing of the osteoarthritic femoral head of the hip joint and proven ability to decrease in subchondral bone edema. Conclusion: Taken together, Intra-articular injection of autologous BM-MSCs represents a safe and feasible treatment tool in patients with hip osteoarthritis during the short term follow up.

Poster Board Number: F-3330

HUMAN MESENCHYMAL STEM CELLS STIMULATE *IN VITRO* MINERALIZATION OF MG63 OSTEOBLAST PRECURSORS

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Background: Bone cells are present in a three dimensional environment and interact with each other, as well as extracellular matrix (ECM), through biochemical and mechanical cues maintaining their specificity and homeostasis of the tissue. MG-63 is a non-mineralizing cell line isolated from human osteosarcoma. In this study, we aimed to determine the effect and influence of cellular communication on the *in vitro* maturation and mineralization. We investigated the interaction of MG63 and human mesenchymal stem cells using both paracrine and direct signaling models. Materials and methods: Human bone marrow was obtained from patients undergoing hip replacement with full ethical approval. Bone marrow stromal cells were isolated through Ficoll gradient followed by adhesion to plastic. MG-63 were obtained from ECACC, UK. After expansion, cells were cultured separately, or in co-culture, with osteogenic medium containing 10 nM dexamethasone, 5 mM β-glycerol phosphate and 50 µg/ml ascorbic acid for 28 days at 37°C, in 5% CO2. Cells were cultured either as high density micromass cultures, within a collagen gel or in monolayer culture. Both direct and indirect signaling was investigated. To investigate paracrine signaling transwell membrane inserts were used to physically separate the cells. Medium was changed every 2-3 days. Results: Results showed that maintaining MSC or MG-63 cells individually in culture was not sufficient to induce mineralization of either cell type within 28 days. When these cells were kept in direct or indirect co-culture, robust mineralization was induced after 21 days of culture. Moreover, results from the indirect co-culture system indicated that mineralization was only observed in MG63 cells and only when they were cultured on a stiff substrate. Discussion: These results suggest that paracrine cell-cell communication from MSC to osteoblast precursor is one of the mechanisms for ECM mineralization in bone cells. Moreover, they also suggest that MSC cells are directly modulating the process, indicating that trophic signals from MSCs may play a key role in bone fracture repair. This could lead to new treatment strategies where MSCs are used to drive and enhance the endogenous response rather than using MSCs as an alternative to osteoblasts.

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DONOR-DEPENDENT DIFFERENCES IN IMMUNE MODULATORY ACTIVITIES OF HUMAN BONE MARROW DERIVED STROMAL CELLS *IN VITRO*

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Human bone marrow-derived stromal cells (BMSCs), also called mesenchymal stem cells (MSCs) have been in clinical use for the last 7 years to treat graft versus host disease and a variety of other immunological abnormalities. To prepare such cells, bone marrow biopsy samples are placed in tissue culture dishes and the plastic-adherent cell population is cultured. Passage 3 or 4 (P3 or P4) cells are stored in frozen aliquots until used. Heterogeneity of the adherent cell population has been noted and studied by a number of investigators using a variety of methods. To look for differences among BMSCs derived from several healthy volunteers, we performed *in vitro* assays of immune modulatory functions of the cells. Methods. We used two *in vitro* assays: 1. The mixed leukocyte reaction (MLR): Peripheral blood mononuclear cells (PBMC) were plated in 96-well plates (150,000 responders/well). Responders were co-cultured with 2500cGy-irradiated stimulator PBMCs (1x105 cells/well). BMSCs were added in the following numbers: 104, 4x104 and 105 cells/well. Culture plates were incubated for 6 days and on the day of harvest 0.5µCi of 3H-thymidine was added to each well for 4 hours. Counts incorporated into DNA were detected with a liquid scintillation counter and reflected lymphocyte proliferation. The effect of BMSCs on the MLR was calculated as the percentage of the mean suppression compared with the proliferative response of the positive control without BMSCs. The experiments were performed at least three times for each variable described. 2. Monocyte/macrophage-derived IL-10 production in co-cultures of BMSCs and two human monocytic cell lines (THP1 and U937) following LPS stimulation: THP-1 or U937 cells (100,000 per well) were plated in 96-well plates. After 3 hours, 25,000 human BMSCs from different donors were added per well and the co-culture was stimulated with phorbol myristate acetate (PMA, 20 ng/ml) to induce the differentiation of monocytic cells into macrophages. After an overnight incubation, the co-culture was stimulated with 1 mg/ml LPS for 6 hours. The supernatants were assayed for IL-10 using the DuoSet IL-10 ELISA kit (R&D). All measurements were performed in quadruplicate. Results. The mixed leukocyte reaction (MLR) is an indicator of the BMSCs' ability to suppress T-cell proliferation. We found that all BMSCs affected T cell proliferation even at the lowest cell number used (10,000/well). Using this number of cells, the suppression varied from 52% to 83% among the 6 volunteers studied. IL-10 induction in monocytes/macrophages reflects the shift that underlies the switch from a pro-inflammatory to an anti-inflammatory state. We found that the BMSC-induced IL-10 concentration varied between 317-1218 pg/ml among the 8 volunteers studied. Interestingly, the same subject who had the strongest T cell suppression also had the highest IL-10 induction, suggesting that his/her BMSCs may have the strongest immuno-modulatory properties in the assays we used. Our results indicate that prescreening BMSCs may be useful in selecting specific lots of cells for use in patients in need of immunosuppression and to determine the number of cells to administer. Further studies are needed to confirm that the *in vitro* assays we used are the best predictors of immunomodulation for optimal cell usage.

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ISOLATION AND EXPANSION OF HUMAN BONE MARROW-DERIVED MESENCHYMAL STEM CELLS AND HUMAN ADIPOSE-DERIVED STEM CELLS IN A SERUM-FREE MEDIUM

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Bone marrow-derived mesenchymal stem cells (BM-MSCs), and their close relative adipose-derived stem cells (ADSCs), are multipotent stem cells capable of immunomodulation, tissue repair and tissue regeneration. BM-MSCs and ADSCs are currently being used clinically for treatment of a variety of indications including inflammatory diseases, cardiac diseases, and repair and regeneration of tissues such as bone, fat and cartilage. For many therapeutic applications, BM-MSCs and ADSCs need to be isolated and expanded ex-vivo in order to generate sufficient cell numbers for a clinical dose. Currently, the majority of researchers and cell therapy companies isolate and expand BM-MSCs and ADSCs in serum-containing formulations. However, researchers and companies are recognizing the need to switch to serum-free culture conditions due to safety and efficacy concerns, and issues with lot-to-lot variability with serum-containing media. While serum-free media has been shown to expand BM-MSCs and ADSCs, isolating BM-MSCs and ADSCs under serum-free conditions has traditionally been problematic. BD has developed a serum-free media formulation, BD Mosaic™ hMSC SF, that can be used to isolate and expand MSCs from bone marrow, and isolate and expand ADSCs from fat. Mosaic consists of a base media formulation, a growth factor supplement, and a surface coating reagent that have been optimized for expansion of BM-MSCs and ADSCs. BM-MSCs were isolated from mononuclear cells from bone marrow aspirates of 3 donors on Mosaic surface coating reagent with Collagen I and IL-17 supplementation. These isolation conditions were tested against a traditional serum-containing media isolation using qualified serum lots. In a second set of experiments, ADSCs were isolated from fat tissue using a similar protocol and also compared to serum-containing media. Both cell types were then expanded in culture in both serum-free and serum-containing media. Colony forming unit-fibroblast (CFU-f) assays showed that BM-MSCs and ADSCs formed colonies in both serum and serum-free conditions demonstrating the ability of Mosaic to support the growth of cells with high proliferative capacity in culture. Data show greater initial numbers of BM-MSCs at P0 in Mosaic and decreased time to 8 population doublings from P0 (12.5 days vs. 16 days, respectively). Although the immunophenotype of Mosaic-isolated MSCs were similar to serum isolated, Mosaic MSCs exhibited more homogeneous expression of CD44. Cells grown in Mosaic and serum-containing media were also both able to differentiate into adipocytes, osteocytes and chondrocytes. In addition, cells grown in Mosaic and serum-containing media were able to suppress T-cell proliferation in CD3/CD28 stimulated peripheral blood mononuclear cells cultures. Results from these experiments suggest that BD Mosaic, which is a serum-free media, is a suitable substitute for serum-containing media for both isolating and expanding BM-MSCs and ADSCs.

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Poster Board Number: F-3334

HUMAN PLACENTA-DERIVED MULTIPOTENT CELLS EFFECTIVELY PROTECT IMMUNOCOMPETENT MICE AGAINST DIABETOGENIC ACTION OF STREPTOZOTOCIN

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Diabetes mellitus (DM) is a chronic disease resulting in high mortality and morbidities including cataract development, renal impairment, and cardiovascular disorders. While the pathological condition of DM is known to be caused by the lack of insulin due to pancreatic beta islet cell destruction, current therapies are not curative and often have considerable side effects. The prevalence of this severe and costly disease is increasing worldwide due to aging and obesity_two known risk factors for the most prevalent type of DM_ thus, more effective therapy is urgently needed. Moreover, once end-organ damage has developed, insulin-directed treatments can no longer reverse these DM-related complications. Thus, we sought to develop better strategies for preventing the progression of DM using human placenta-derived multipotent cells (PDMCs), an ethically compliant population of human mesenchymal stem cells with strong immunomodulatory properties which are easily expanded *ex vivo*, a critical issue in clinical applications. To explore this strategy, we developed an in vivo model of DM using streptozocin (STZ) in immunocompetent mice (C57BL/6 strain) rather than immunocompromised mice to better mimic physiologic conditions. Mice developed DM as manifested by hyperglycemia within 48 hours after STZ treatment and were subsequently subjected to multiple human PDMC xeno-transplantation. Diabetic mice treated with human PDMCs from three independent donors did not show signs of immune rejection towards the xenogeneic cells, but rather demonstrated reduced blood glucose level and increased body weight when compared with control diabetic mice. Importantly, we found that the therapeutic effect of multiple human PDMC treatment was persistent in STZ-induced diabetic mice for at least three months. Histologic examination demonstrated that pancreatic islets which were obliterated in STZ-treated diabetic mice recovered to some extent after human PDMC treatment. RT-PCR assay further confirmed that the expression levels of pancreatic beta cell genes such as Ins2 and Nkx6.1 were enhanced in human PDMC-treated diabetic mice when compared with that in control diabetic mice, implying that some recovery of beta islet cells with human PDMC treatment may be ongoing. Taken together, these findings suggest that human PDMC transplantation may be a safe and promising strategy against DM.

Poster Board Number: F-3335

ACQUISITION OF HUMAN ALVEOLAR BONE-DERIVED STROMAL CELLS USING MINIMALLY IRRIGATED IMPLANT OSTEOTOMY

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Objectives: Implant osteotomy yields a substantial amount of bone in the form of bone chips entrapped within drill flutes, and can provide a promising cell source for tissue engineering. The aims of this study were to isolate human alveolar bone-derived stromal cells (hABCs) obtained during implant osteotomy, and to evaluate osteogenic differentiation capacity of hABCs. Materials and methods: Bone chips were obtained by minimally irrigated implant drilling technique from ten human donors. Isolated cells were studied with respect to their colonyforming efficiency and surface marker expression by immunofluorescence staining and fluorescence-activated cell-sorting analysis. To verify the differentiation activity, in vitro osteogenic and adipogenic gene expressions were evaluated by reverse transcription polymerase chain reaction, and in vitro formation of mineralized nodule and adipocytes was also evaluated. *In vivo* bone forming activity was assessed by ectopic transplantation in immunocompromised mice (n=5). Result: hABC population was present in the isolated cells. Upon hABC transplantation, significant ectopic bone formation was induced with the characteristics of fully matured bone tissue. Conclusion: The data support the feasibility of using hABCs as a source of cells for dentoalveolar bone tissue reconstruction. The cell source has advantage that hABCs can be easily acquired during implant surgery. This work was supported by Mid-career Researcher Program through an NRF grant funded by the MEST (No. 2009-0078884), and by a grant of the Korea Health Technology R&D Project, Ministry of Health, Wellfare & Family Affairs, Republic of Korea (no. A100443).

Poster Board Number: F-3336

TARGETING INSULIN RESISTANCE VIA THE IMMUNE MODULATION OF HUMAN CORD BLOOD-DERIVED MULTIPOTENT STEM CELLS BY THE STEM CELL EDUCATOR THERAPY

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The prevalence of type 2 diabetes (T2D) is increasing worldwide, highlighting the need for a better understanding of the pathogenesis of the disease and the development of innovative therapeutic approaches for the prevention and cure of the condition. Mounting evidence points to the involvement of immune dysfunction in insulin resistance in T2D, suggesting that immune modulation may be a useful tool in treating the disease. We developed an innovative procedure for Stem Cell Educator therapy in which a patient's

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blood is circulated through a closed-loop system that separate lymphocytes from the whole blood and briefly co-cultures them with adherent human cord blood-derived multipotent stem cells (CB-SCs), and returns the educated lymphocytes (but not the CB-SCs) to the patient's circulation. In an open-label, phase1/phase 2 clinical trial (n = 15) have demonstrated that Stem Cell Educator therapy reverses autoimmunity and promotes regeneration of islet beta cells in long-standing diabetics with moderate or severe type 1 diabetes. In an open-label, phase1/phase 2 study, patients (n = 25) with T2D received one treatment with the Stem Cell Educator. Median age was 50 years (range, 29 to 66), and median diabetic history was 9 years (range, 1 to 25). Notably, clinical findings indicate that T2D patients achieve improved metabolic control and reduced inflammation. Median glycated hemoglobin (HbA1C) was significantly reduced from 8.47% ± 0.99 at baseline to 7.87% ± 1.07 at 4 weeks post treatment (p = 0.022), and to 7.1% ± 0.6 at 12 weeks post treatment (p = 1.6E-05). More than 80% of subjects achieved the <7% standard recommended by the American Diabetes Association (ADA). Homeostasis model assessment (HOMA) of insulin resistance (HOMA-IR) and HOMA-pancreatic islet beta-cell function (HOMA-B) demonstrate that insulin sensitivity have been improved post treatment. Mechanistic studies revealed that Stem Cell Educator therapy can correct the immune dysfunction, as demonstrated by balancing the Th1/Th2/Th3 cytokine productions. Thus, Stem Cell Educator therapy is safe, and in individuals with moderate or severe T2D, a single treatment produces lasting improvement in metabolic control. Mechanistic studies revealed that CB-SCs express autoimmune regulator (Aire). Knockdown of Aire indicate that Aire is involved in immune modulation and induction of immune tolerance following Stem Cell Educator therapy. During *in vitro* co-cultures, CB-SCs attached to interior surfaces in the device present secreted and cell-surface signaling molecules (e.g., nitric oxide and PD-L1) to passing lymphocytes, and only the CB-SC-modulated autologous lymphocytes are returned to the subjects. Therefore, Stem Cell Educator therapy is very safe approach without any adverse events in all participants. Thus, our findings indicate that Stem Cell Educator therapy can modulate immune dysfunction, increase insulin sensitivity, overcome chronic metabolic inflammation and insulin resistance, and promote the regeneration of islet beta cells, without the safety, regulatory and ethical concerns associated with conventional stem cell-based approaches.

Poster Board Number: F-3337

GENE MODIFIED HUMAN ADIPOSE-DERIVED MESENCHYMAL STEM CELLS OVEREXPRESSING ANTI-INFLAMMATORY CYTOKINES DAMPEN INFLAMMATORY RESPONSES AND PROMOTE FUNCTIONAL RECOVERY IN EXPERIMENTAL AUTOIMMUNE DEMYELINATION

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The anti-inflammatory cytokines interleukin (IL)-10 and IL-4 play an important role in regulating disease severity in experimental autoimmune encephalomyelitis (EAE), the predominant animal model of multiple sclerosis (MS). This is illustrated by the increased expression of IL-4 and IL-10 mRNA during periods of disease remission and the severity of EAE in mice deficient in these cytokines. Exogenously delivered IL-4 and IL-10 can attenuate clinical disease; however continuous doses are required as a consequence of the short half-life of cytokines *in vivo*. Apart from their broad immunomodulatory and tissue reparative properties, stem cells are capable of homing to sites of inflammation and therefore represent promising tools for the delivery of therapeutic molecules. Previously studies in our laboratory have identified human adipose-derived mesenchymal stem cells (Ad-MSCs) as a promising source of MSCs for therapeutic applications. Ad-MSCs can inhibit the proliferation of lymphocytes in vitro and suppress clinical and pathological signs of disease in EAE mice. Here, we report on the effect of Ad-MSCs engineered to overexpress anti-inflammatory cytokines in mice with EAE. Ad-MSCs transduced with a bicistronic lentiviral vector encoding either human IL-10 or mouse IL-4 and enhanced green fluorescent protein were found to express stably high levels of transgene. Importantly, gene modified Ad-MSCs maintained their differentiation potential, cell surface phenotype, expression of homing receptors and immunosuppressive properties. Transplantation studies revealed that Ad-IL10-MSCs could prevent or significantly delay the development of EAE when administered during the priming phase of disease, reducing T-cell proliferative responses and pro-inflammatory cytokine secretion. Ad-IL10-MSCs inhibited dendritic cell function in co-culture studies, suggesting that the mechanism of action may involve inhibition of antigen presentation and T-cell activation. Likewise, transplantation of Ad-IL4-MSCs reduced EAE severity, shifting the cytokine response from pro- to anti-inflammatory. Together our results demonstrate that gene modified Ad-MSCs may offer a novel mode for the treatment of a variety of inflammatory and degenerative diseases.

Poster Board Number: F-3338

3D-CULTURE ENHANCES THE HOMING CAPACITY OF HUMAN MESENCHYMAL STEM CELLS TO THE INFARCTED MYOCARDIUM IN MICE

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Transplantation of human mesenchymal stem cells (hMSCs) has been proposed as a strategy for cardiac repair following myocardial damage. However, poor cell homing ability of *ex vivo* expanded hMSCs after transplantation has limited the reparative capacity of these cells in vivo. We hypothesized that 3D-culture improved the homing capacity and therapeutic effect of hMSCs. Here, we used a hanging drop protocol to prepare hMSCs as spheroid. After the hanging drop culture procedure, the regularly cultured expanded (adherent) hMSCs changed to a smaller volume, a higher colony forming efficiency and formed larger colonies. In addition, molecular studies revealed that cells from spheroids showed up-regulated expression of stem-related genes such as OCT4, SOX2 and NANOG, migration related genes such as CCR1, CCR2, CCR3, CX3CR, CXCR4 and CD49b, and anti-inflammatory cytokine genes such as IL-10, IL-11 and IL-13. To examine the homing capacity of hMSCs after sphere culture, 2×106 cells were intravenous injected in mice following myocardial infarction, and regularly cultured adherent hMSCs were used as control. One week later, the amount of hMSCs recovered from the heart in the spheroid hMSC group was greater than that from the regular adherent culture group. Moreover, the infarct size was significantly smaller in the spheroid hMSC group compared to the adherent hMSC group. Cardiac ultrasound analysis showed increased ejection fraction and fractional shortening in the spheroid hMSC group compared to the adherent hMSC group ($P<0.05$), suggesting an improved left ventricular function. In conclusion, our results suggest that spheroid culture is an effective method to increase the homing capacity and therapeutic effects of hMSCs after *ex vivo* expansion.

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TRANSPLANTATION OF HUMAN DENTAL PULP STEM CELLS AMELIORATES DIABETIC POLYNEUROPATHY IN MICE

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Diabetic neuropathy is the most common complication of diabetes. We have previously shown that the transplantation of cultured endothelial progenitor cells or bone marrow-derived mesenchymal stem cells ameliorated diabetic neuropathy in rats. Human dental pulp stem cells (hDPSCs) which are a sort of mesenchymal stem cells located in the dental pulp cavity are expected as a source of regenerative medicine, since hDPSCs can be isolated from wisdom tooth extraction or premolar extraction for orthodontic reasons. In this study, we investigated the therapeutic potential of hDPSCs transplantation on diabetic polyneuropathy in streptozotocin (STZ)-induced diabetic nude mice. Methods: We collected human impacted third molars from 4 adults (13-23 years of age) at Aichi-Gakuin University hospital. Written informed consent was obtained from each donor. Dental pulp was extracted by cutting teeth and hDPSCs were isolated by collagen digestion method and cultured as previously described. Identification of hDPSCs was analyzed by surface makers and differentiation capabilities. Diabetes was induced by an intraperitoneal injection of STZ in 6 week-old BALB/cAJcl-nu/nu mice. Eight weeks after STZ injection, hDPSCs (1×10^5 cells/limb) were transplanted into unilateral hindlimb skeletal muscles of normal and diabetic mice. Saline was injected into the other side as control. Sciatic blood flow (SNBF), sciatic motor /sensory nerve conduction velocity (MNCV/SNCV) and current perception threshold (CPT) were evaluated 4 and 16 weeks after the transplantation of hDPSCs. Immunohistological analysis of hindlimb skeletal muscles was also performed at the end of the experiments. Results: Flow cytometric analyses showed the positive stainings of CD29 and CD90 and negative stainings of CD34 and CD45. hDPSCs differentiated into osteoblasts and adipocytes by each induction media. Diabetic mice showed significant reductions in SNBF, MNCV and SNCV and increase in CPTs in the control side compared with normal mice. Transplantation of hDPSCs significantly ameliorated the impaired SMBF, MNCV, SNCV and CPTs in the hDPSCs-injected side of diabetic mice. Immunohistological study revealed that the transplanted hDPSCs were located around the muscle bundles and not differentiated into adipocytes nor osteoblasts. Conclusion: We have demonstrated the efficacy of hDPSCs transplantation for diabetic neuropathy without any adverse effects, suggesting that the transplantation of hDPSCs could be a new strategy for the treatment of diabetic neuropathy.

Poster Board Number: F-3340

COMPARISON OF CTX0E03 (CTX) DRUG PRODUCTS IN A MURINE MODEL OF HIND LIMB ISCHEMIA

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The development of cell therapies for clinical use may require establishing different formulation strategies for the shipping and storage of a final drug product. ReNeuron has established the *CTX* neural stem cell line, currently in a Phase I clinical trial for treatment of chronic disability post-stroke (the PISCES trial, clinicaltrials.gov #NCT01151124). The *CTX* cell line has also shown dose-related efficacy in the pre-clinical model of severe peripheral arterial disease using murine hind limb ischemia model (Presented at Scientific Sessions of American Heart Association, 2010). The current formulation of *CTX* as a drug product is a freshly harvested cell preparation with a 96 hour shelf life when stored at 4°C. An alternative and more cost-effective approach would be to use a stem cell formulation that could be delivered to and stored at the clinical site frozen. Thawing and possibly cell dilution would then be the only manipulation at site. To determine if altering the product formulation affects efficacy, a comparison study was conducted using fresh and frozen cell formulations in two different, commercially available, excipients. A number of functional measures were taken over a 21 day time period. Hind limb ischemia was performed, as described in Madeddu et al 2006, in CD-1 mice by permanent ligation of the femoral artery followed by intra-muscular injection of a vehicle (Hypothermosol®-HTS or Cryostore-2®-CS2, [Biolife Solutions]) or a cell product (CTX freshly prepared in HTS, CTX frozen in HTS, CTX frozen in CS2) into the adductor muscle of the ischemic limb immediately after ligation with n=8 in all groups. Frozen cells were thawed shortly prior to injection and diluted in the appropriate excipient without further processing. The injected cells had a final concentration of 10,000 cells/µl for a total of 300,000 cells per animal. Animals were monitored for tissue necrosis daily and had multiple readings of blood flow using laser Doppler. At the end of the study (day 21) tissue oxygen pressure readings were made prior to sacrifice. There were similar outcomes between the two vehicle treated groups in the tissue necrosis and laser Doppler measures. The CS2 treated group had lower tissue oxygen pressure compared to the HTS treated group($p<0.001$, t-test). Compared to the vehicle treated groups, cell treatment significantly reduced the development of necrosis ($p<0.01$, Log Rank test), increased blood flow as monitored by laser Doppler ($p<0.001$, 2 way ANOVA) and increased tissue oxygen pressure ($p<0.001$, 1 way ANOVA). There were no significant differences between the cell treatment groups. These data demonstrate that altering methods of formulation does not affect the efficacy of the CTX neural stem cell line in the murine model of hind limb ischemia. The ability to develop a frozen stem cell product that has the potential to be mass produced in a central facility shipped to clinical sites and held without further regulated processing prior to use increases the practical application and very substantially lowers the cost of this class of therapeutics.

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Poster Board Number: F-3341

ADIPOSE-DERIVED STEM CELLS FROM HUMAN/ RAT/MOUSE AS A NEW TOOL FOR THE TREATMENT OF SYSTEMIC SCLEROSIS.

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POTENTIAL OF ADIPOSE-DERIVED STEM CELLS FROM HUMAN/ RAT/MOUSE AS A NEW TOOL FOR THE TREATMENT OF SYSTEMIC SCLEROSIS. Accumulating evidence suggests that adipose tissue-derived mesenchymal stem/stromal cells (ASC) promote tissue regeneration and regulate immune reaction. We have shown that low (2%) serum cultured ASC (LASC) was superior to high (20%) serum cultured ASC (HASC) in terms of their therapeutic effects in various animal models including hind limb ischemia, acute kidney injury, glomerulonephritis and xenotransplantation. The aim of the present study was to develop a novel cell therapy using LASC for patients with systemic sclerosis (SSc). We evaluated the therapeutic effects of LASC as well as their safety using *in vivo* models. Firstly, the therapeutic effect of ASC was evaluated in a rat model of skin ulcer, which is one of the major complications of SSc patients. Rat LASC secreted higher levels of VEGF, HGF and KGF as compared to rat HASC. A skin ulcer model was made in rats by removing the total skin of the back, and rat LASC, rat HASC or vehicle was injected to the skin around the ulcer. The level of HGF was increased in the injection site of LASC, and LASC decreased the size of skin ulcer more rapidly than HASC or vehicle. Next, the therapeutic effect of ASC was evaluated in two kinds of mouse models of SSc. One model was induced in BALB/c mice by repeated subcutaneous injection of bleomycin for three weeks. Mice LASC (0.3×106 cells/body), LASC pretreated with interferon gamma (IFN) in flask (LASC-IFN) (0.3×106 cells/body), or vehicle were injected via tail vain on days 6 and 13. Mouse LASC suppressed the level of anti-nuclear antibody generated in this model. Pretreatment with IFN further decreased the level of anti-nuclear antibody. Moreover, mouse LASC-IFN significantly decreased the thickness of the skin. The other SSc model was induced by passive transfer of pathogenic CD4 positive T cells. BALB/c mice were given similarly by repeated injection of bleomycin, spleen cells were taken and CD4 positive cells were collected. These cells (1.0×106/body) were transferred to nude mice, which developed symptoms of SSc including skin fibrosis. Mouse LASC (0.5×106 cells/body), LASC-IFN (0.5×106 cells/body), or vehicle were given intravenously on days 0, 7, 14, and the therapeutic effect was evaluated on day 21. Mouse LASC (0.5×106 cells/body) as well as LASC-IFN significantly decreased thickness of the skin. Lastly, an experiment to assess the possible risk of tumorgenesis by LASC was performed. Nude rats (n=9) was administered with LASC into the subcapsular space of the kidney (1.0x107 cells) on day 0 and via the tail vein (5x106 cells/body) on days 1, 8, and 15. Six months later rats were euthanized and organs were obtained. Histological analysis revealed that none of these rats developed malignant tumors in any organs. In conclusion, the present study demonstrates that LASC has a great potential as a tool for the novel cell therapy to the patients with systemic sclerosis.

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IMMUNOSUPPRESSION AND THERAPEUTIC POTENTIAL OF BONE MARROW-DERIVED MESENCHYMAL STEM CELLS IN NON-OBESE DIABETIC MOUSE MODEL: AN MHC-INDEPENDENT AND CONTACT-DEPENDENT PROTECTIVE MECHANISM TRIGGERED BY PROINFLAMMATORY CYTOKINES

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The beneficial effects of mesenchymal stem cells (MSCs) in preventing autoimmune diabetogenesis and in improving the survival of islet grafts are attributed by their direct or indirect actions in suppressing immune or inflammatory responses through contact and non-contact pathways and in promoting islet survival and function by enhancing graft revascularization, respectively. However the therapeutic potential of syngeneic MSCs in autoimmune diabetes has not been clarified yet. We investigate the hypothesis that bone marrow-derived (BM)-MSCs prevent the development of autoimmune diabetes in non-obese diabetic (NOD) mice and prolong the survival of islet grafts in a syngeneic manner. Our results demonstrate that cell-cell contact is required for BM-MSCs-mediated immunomodulatory effects and those responses are mouse strain independent. Regulatory pathways including nitric oxide synthase, proinflammatory cytokines and PD-L1 are involved in this BM-MSCs-based immune non-responsiveness. Moreover, BM-MSCs promote the induction of regulatory T cells and myeloid-derived suppressor cells, and inhibit the development of dendritic cells and T cells. Interestingly, an enhanced adhesion between proinflammatory cytokine-triggered BM-MSCs and hematopoietic cells may participate in this immunosuppression. Notably, transferred syngeneic BM-MSCs tend to migrate to pancreatic lymph node and significantly delay the disease onset in NOD/SCID recipients which were pre-transferred with pathogenic lymphocytes. Strikingly, those BM-MSCs also efficiently prolong the survival islet grafts, supporting the therapeutic potential of autologous BM-MSCs in autoimmune diabetes.

Poster Board Number: F-3343

UMBILICAL CORD MESENCHYMAL STEM CELL AMELIORATES DERMATOPHAGOIDES FARINAE INDUCED ATOPIC DERMATITIS LIKE SKIN LESIONS IN NC/N_{GA} MICE BY INHIBITING MAST CELL DEGRANULATION.

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Atopic dermatitis (AD) is a clinical syndrome that is characterized by pruritic skin lesions that are distinguished by infiltrating lymphocytes, macrophage and granulated mast cells. Umbilical cord-blood derived mesenchymal stem cell (USC) transplantation has been widely used in the treatment of a variety of diseases due to their advantages such as abundant resources, low immunogenicity and large *ex vivo* expansion capacity. They have been found to have immunosuppressive properties and the ability to modulate angiogenesis and tissue repair by in vitro and animal studies. Clinical

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trials have examined the utility of these cells in autoimmune and inflammatory conditions. USC has been shown to have immunoprivileged properties, but its effect on AD has not been examined. In this study, the immunomodulatory effects of USC, using dermatophagoides farinae (Df)-induced AD-like skin lesions in NC/Nga mice, were investigated. The clinical scores were reduced significantly by the intrasubcutaneous transplantation with both USC and MDP pretreated USC (MDP-USC) at 2X105 cells /mouse. Histological analysis of the skin also revealed that transplantation of USC and MDP-USC significantly reduced the inflammatory cellular infiltrate, including mast cells. Moreover, USC and MDP-USC decreased not only the degranulation of mast cell in AD-like skin lesions but also the levels of serum IgE in AD mouse model. Under in vitro conditions of co-culture, USC markedly inhibited the degranulation of mast cells derived from umbilical cord blood. Remarkably, mast cell degranulation was further suppressed in the presence of MDP-USC, which was completely rescued by down-regulation of PGE2 synthesis and neutralization of TGF-β1. These results suggested that USC and MDP-USC inhibited the development of Df-induced AD-like skin lesions in NC/Nga mice through inhibition of mast cell degranulation, which was mediated by the concerted action of USC-derived PGE2 and TGF-β1. Therefore, our results indicated that USC might be a useful immunomodulatory agent for the treatment of human AD.

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FUNCTIONAL RECOVERY OF WISTAR RATS WITH TRAUMATIC SPINAL CORD INJURY AFTER *IN SITU* ADMINISTRATION OF HUMAN UMBILICAL CORD MESENCHYMAL STROMAL CELLS

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There is still no effective therapy for spinal cord injury (SCI). Cell transplantation is a promising strategy to treat this condition. Adult stem cells, such as human mesenchymal stromal cells (hMSCs) are a potential source for reducing injury and promoting recovery of damaged tissues, such as spinal cord. An evaluation was made of the efficacy of hMSCs from human umbilical cord vessels to promote functional recovery when transplanted in rats after induced contusion SCI. Female Wistar rats were submitted to spinal injury with a MASCIS impactor and divided into 4 groups: control, surgical control, SCI and a cell-treated lesion group. hMSCs of the human umbilical cord vessels were transplanted in three experiments: a) 1 h post surgery, into the injury site at a concentration of 3x105 cells diluted in 10 µL 0.9% NaCl (N=10 per group); b) into the cisterna magna, 24 hours after lesion at the same concentration diluted in 150 µL 0.9% NaCl (N=6 per group) and c) into the cisterna magna, 9 days after lesion at the same concentration diluted in 150 µL 0.9% NaCl (N=6 per group). The transplanted animals were immunosuppressed with cyclosporin-A (10 mg/kg per day). The Basso, Beatie and Bresnahan (BBB) scale was used to evaluate motor behavior of the lower members. The BBB scale ranges from 0 points (total paralysis) to 21 (normal movement). The injury site was analyzed with the following immunofluorescent markers: human-specific anti-NUMA, to detect the human transplanted cells; anti-NG2, to label the oligodendrocytes; synaptophysin as a neuronal marker; and GFAP, to label the astrocytes. Transplanted cells survived in the injury area for 6 weeks after the procedure. The animals that received hMSCs one hour after injury at the end of the sixth week reached a score of 18.8 points. This result was significantly different from the control injured rats without treatment, which showed a mean score of 12.7 points ($p<0.05$). In the experiments with cells transplanted 24 hours and 9 days post injury, the treated rats did not exhibit significant motor recovery when compared with the non treated injured rats. The results demonstrate that: a) stem cell transplantation was effective for functional recovery of SCI only when performed on the lesion site 1 h after injury, as compared with cell administration in the cisterna magna 24 hours and 9 days after injury; b) the transplanted human cells migrated and survived in the injured area for 6 weeks post injury procedure when administered in the cisterna magna; c) the cells survived in the injury site when administered directly and d) the hMSCs did not differentiate into glial cells or neurons, suggesting that functional recovery was due to other factors promoting neuroprotection. Despite the absence of detection of the differentiation of the transplanted cells, it can be concluded that the transplantation of hMSCs promotes functional recovery after SCI when performed 1 hour after injury directly at the injury site.

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GENE EXPRESSION OF AXONAL GUIDANCE/ REPULSION AND NEUROTROPHIC FACTORS OF CANINE BONE MARROW STROMAL CELLS

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Introduction: Recently, the clinical application of spinal cord regenerative therapy using bone marrow stromal cells (BMSCs) has become an option for the treatment of severe spinal cord injury. However, the mechanism of spinal cord regeneration by BMSC transplantation is not fully understood. A previous study demonstrated that BMSCs guided axons to spinal cord injury sites in rat and mouse models. It has been speculated that axonal guidance or neurotrophic factors secreted from BMSCs might be related to spinal cord regeneration. Unfortunately, information about these factors that are secreted from the BMSC is limited. Therefore, we investigated the mRNA expression levels of axonal guidance/repulsion and neurotrophic factors of BMSCs in dogs as large animal models. Materials and Methods: Six healthy beagles were used in the present study. Bone marrow was drawn from the humerus under general anesthesia. BMSCs were isolated by density gradient centrifusion and then static-cultured in an incubator at 5% CO2 and 37°C using α-MEM with 10% FBS. Canine dermal fibroblasts (DFBs) were used as negative controls. After one passage, total RNAs were extracted from BMSCs and DFBs using TRIZOL® at 3 days of culture. The first-strand cDNA synthesis was carried out with 500 ng total RNA using PrimeScript® RT Master Mix. Real-time PCRs were then performed with 2 µl first-strand cDNA synthesis using SYBR® Premix Ex Taq II and primers specific to canine NTN1, NTN3, NTN4, NTN5, NTNG1, NTNG2, SEMA3A, SEMA4D, SEMA5A, SEMA6D, EFNA1, EFNA3, EFNA4, EFNB2, EFNB3, SLIT1, SLITRK1, SLIT2, NGF, and BDNF. The results were analyzed using TP900 DiceRealTime v4.02B. The amplification of GUSB from the same amount of cDNA served as an endogenous control. Statistical analysis was performed using

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StatMateIV software and Student's t-test. P values less than 0.05 were considered significant. Results: The mRNA expression levels of NTN1, NTN3, NTNG2, SEMA4D, SEMA5A, EFNA3, and EFNA4 were significantly higher in BMSCs than in DFBs. In contrast, no significant differences in mRNA expression levels of NTN5, SEMA6D, EFNA1, EFNB2, EFNB3, SLIT1, and NGF were observed. The mRNA expression levels of NTN4, NTNG1, SEMA3A, SLITRK1, SLIT2, and BDNF in BMSCs were significantly lower than those in DFBs. EFNB3 mRNA expression was not detected in either BMSCs or DFBs. Discussion: To our knowledge, there have been no reports on the mRNA expression levels of NTN3, NTN4, NTN5, NTNG1, NTNG2, SEMA3A, SEMA4D, SEMA5A, SEMA6D, EFNA1, EFNA3, EFNA4, EFNB2, EFNB3, SLIT1, SLITRK1, and SLIT2 in BMSCs. In the present study, the mRNA expression levels of NTN1, NTN3, SEMA4D, SEMA5A, EFNA3, and EFNA4 in BMSCs were significantly higher than those in DFBs. NTN1 and NTN3 are genes of axonal guidance factors. SEMA4D, SEMA5A, EFNA3, and EFNA4 are genes of factors related to axonal guidance, although these factors are axonal repulsion factors. Therefore, these factors may be important for axonal guidance in injured spinal cords. On the other hand, the mRNA expression levels of SEMA3A, SLITRK1, and SLIT2 were significantly lower than those in DFBs. SEMA3A, SLITRK1, and SLIT2 are genes of axonal repulsion factors that hinder axon attraction. This suggested that the lower levels of these factors might be beneficial to axonal guidance. Further investigation of the secretion levels of netrin-1 and -3, semaphorin-4D and -5A, and ephrin-A3 and A4 from BMSCs is needed to clarify that these factors are related to spinal cord regeneration.

Poster Board Number: F-3346

MESENCHYMAL STEM CELLS TRANSPLANTATION INTO THE KNEE JOINTS OF HARTLEY STRAIN GUINE PIG WITH SPONTANEOUS OSTEOARTHRITIS

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Introduction:Mesenchymal stem cells (MSCs) can differentiate into various connective tissue cells. Several techniques have been used for the clinical application of MSCs in articular cartilage repair, however, there are many issues associated with the selection of the scaffold material, including its ability to support cell viability and differentiation and its retention and degradation *in situ*. The application of MSCs via a scaffold also requires a technically demanding surgical procedure. The aim of this study was to test the outcome of intra-articular transplantation of mesenchymal stem cells suspended in hyaluronic acid (HA) in the knee joints of Hartley strain guinea pigs with spontaneous OA. Methods:Commercially-available human MSCs were cultured, labeled with carboxyfluorescein diacetate succinimidyl ester (CFDA-SE), suspended in either phosphate-buffered-saline (PBS) or HA, and injected into the knee joints of 7-month-old animals. The control animals were injected with either PBS or HA alone. The animals were sacrificed at 1, 3,and 5 weeks post-transplantation and the knee joints harvested and fluorescent microscopic analysis was performed. Macroscopic, Histological and immunohistochemical analysis were performed at 5 weeks post transplantation. Results:Clinically, all guinea pigs tolerated the xenogenic MSCs injection, and there was no evidence of local inflammation, joint effusion, or unloading of the joint resulting from the cell treatment. At 5 weeks post-transplantation, partial cartilage repair was noted in the HA-MSC group but not in the other groups. Examination of CFDA-SE-labeled cells demonstrated migration, differentiation and proliferation of MSC in the HA-MSC group. There was strong immunostaining for type-II collagen around both residual chondrocytes and transplanted MSCs in the OA cartilage. On the other hands, a few labeled cells were found within the cartilage at 1 week post-transplantation of PBS+MSC. The labeled cells gradually disappeared from the cartilage at 3 and 5 weeks post-transplantation Conclusion:This scaffold-free and technically un-demanding technique appears to result in the regeneration of articular cartilage in the spontaneous OA animal model. Our study has certain limitations.These include;(i) the use of non-physiologic condition for xenogeneic MSCs transplantation;(ii) lack of re-examination of optimum timing of injection during OA progression;(iii) the use of HA only instead of including another compound with molecular weight similar to HA and so on. Although further examination areneeded that include the above points, the findings suggest that intra-articular injection of a mixture of MSCs-HA is a potentially beneficial for OA

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IN VIVO OSTEOGENESIS OF CANINE MESENCHYMAL STEM CELLS DERIVED FROM BONE MARROW, ADIPOSE AND DERMAL SKIN TISSUES

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Canine mesenchymal stem cells (cMSCs) have generated a great interest as a promising source for cell based bone regenerative medicine. The present study compared the *in vivo* osteogenesis of cMSCs derived from bone marrow (cBM-MSCs), adipose tissue (cA-MSCs) and dermal skin tissue (cDS-MSCs) of a single donor, following their transplantation into BALB/c-nu mice. All cMSCs were isolated from a two-years-old female beagle under standard surgical procedures, and then cultured in A-DMEM with 10% FBS at 38.5°C in a humidified atmosphere of 5% CO2 in air. Isolated cells were characterized by their morphology, CD markers profile (CD45-, 90+ and 105+), cell cycle status, and differentiation ability into osteocytes, adipocytes and chondrocytes following protocols described previously. All three kinds of cMSCs displayed a fibroblast-like morphology, and positively expressed CD markers, such as CD90+ and 105+, but negative for CD45- by immunofluorescence staining. Cell cycle analysis of cMSCs by flow cytometry after 10 µg/ml propidium iodide (PI) staining showed cDS-MSCs with lowest percentage of cells at G0/G1, where as BM-MSCs had the highest. The results of cytochemical staining (Alizarin red S for osteocyte, Oil red O for adipocyte and Alcian blue for chondrocyte) demonstrated the in vitro differentiation capacity of all three kinds of cMSCs, but the rate of differentiation was varied between them. The abilities for osteogenesis and adipogenesis were higher in cA-MSCs than other cells, whereas chondrogenic capacity of cBM-MSCs and cA-MSCs was higher than that of cDS-MSCs. cMSCs of different origins were compared for *in vivo* osteogenesis with demineralized bone matrix (DBM; Osteotech, OK, USA) and transplanted at a concentration of 1x106 cells into the subcutaneous spaces of 9 weeks old BALB/c-nu mice. The mice were subjected for radiographic analysis after one and two months of implantation to examine the calcification. cA-MSCs and contrary to the in vitro observations, cDS-MSCs also displayed higher *in vivo* osteogenesis ability than cBM-MSCs, and these results were supported by Alizarin red S staining using

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cryosectioned tissue of recovered scaffolds. The differences could be related with cell proliferation capacity of cDS-MSCs as they possessed low numbers at G0/G1 phase. Based on our findings, it is concluded that cDS-MSCs and cA-MSCs could become valuable alternative sources to cBM-MSCs for applications in bone regeneration.

Poster Board Number: F-3348

MULTIPOTENT MESENCHYMAL STROMAL CELLS MODULATE BENEFICIAL AND PATHOGENIC T CELL RESPONSES TO THEILER'S MURINE ENCEPHALOMYELITIS VIRUS INFECTION

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Multipotent mesenchymal stromal cells (MSCs) isolated from adult human bone marrow demonstrate regulatory immunomodulatory properties towards a number of effector immune cell types. Specifically MSCs have been shown to inhibit proliferation and activation of effector T cells underlying the rationale for the therapeutic use of MSCs in re-establishing tolerance in autoimmune diseases or following transplantation. Here we investigate a role for MSCs administered in a mouse model of viral infection in which T cells serve juxtaposing roles in both mediating early viral clearance and later driving a demyelinating disease of the central nervous system (CNS) with similarities to multiple sclerosis in humans. Theiler's murine encephalomyelitis virus (TMEV) infection induces an acute encephalomyelitis attack in SJL/J mice that is rapidly though incompletely cleared by CD8+ T cells. The chronic, persistent infection leads to a delayed inflammatory demyelinating disease in which oligodendrocyte damage is caused by virus-specific host CD4+ T cells that cross react with a self epitope. MSCs injected IV early after TMEV infection inhibited T cell-mediated viral clearance as demonstrated by increased viral titers in the CNS as well as decreased lysis of virus-loaded cells injected *in vivo* seven days after infection. MSC-treated mice lost more body weight than control-treated animals further suggesting exacerbated encephalitis. Analysis by flow cytometry revealed CD8+/CD44+ effector T cells were decreased in the CNS and increased in the spleen seven days after infection suggesting a retention of cells in the peripheral immune organs. CD8+ effector T cell numbers in the spleen returned to control levels by 42 days post-infection though numbers in the CNS were still below that of controls. MSCs administered 35 days post-infection during the latent, chronic viral infection likewise increased viral titers in the CNS and decreased delayed type hypersensitivity (DTH) CD4+ T cell responses to a viral peptide *in vivo* seven days after injection. We next asked whether MSC-mediated inhibition of viral clearance or control of latent viral infection altered TMEV-induced demyelinating disease. MSCs injected early after viral infection accelerated disease onset as measured by a standardized scale of clinical and motor deficits. Furthermore MSCs exacerbated clinical disease score, an effect that was sustained at least 200 days post-onset. Assaying *in vivo* CD4+ T cell responses to myelin epitopes by DTH, we observed increased proteolipid protein (PLP) responses 84 days post-infection. Flow cytometric analysis after established demyelinating disease also revealed increased effector T cells in the CNS. Although we found that MSCs injected later during the latent, chronic viral infection inhibited immune responses and increased viral load, there was no change in clinical disease score or incidence. DTH assays likewise revealed no change in CD4+ T cell responses to PLP *in vivo* suggesting MSC administration at disease onset neither alleviated nor exacerbated demyelinating disease. The results here support the work of others demonstrating that MSCs produce rapid effects on immune responses *in vivo*. This is the first evidence for MSC-induced suppression of T cell-mediated viral clearance and maintenance of a latent viral infection. Our work also suggests early inhibition of viral clearance can exacerbate the later development of TMEV-induced demyelinating disease.

Poster Board Number: F-3349

REPEATED MESENCHYMAL STEM CELL INJECTION IMPROVES RADIATION-INDUCED PROCTITIS IN PIG

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The management of proctitis in patients who have undergone very high-dose conformal radiotherapy is extremely challenging. The fibrosis-necrosis, fistulae, and hemorrhage induced by pelvic over-irradiation have an impact on morbidity. Augmenting tissue repair by the use of mesenchymal stem cells (MSCs) may be an important advance in treating radiation-induced toxicity. Using a pre-clinical pig model, we investigated the effect of autologous bone marrow derived-MSCs on high-dose radiation-induced proctitis. For this, 7 pigs received a high dose of X-ray (20-27Gy), delivered to the rectum. 4 pigs received intravenous administrations of autologous MSCs (2.106 MSCs/kg): 2 pigs with 2 injections and 2 pigs with 3 injections on days 27, 34 and 41 post-irradiation. Immunostaining and real-time PCR analysis were used to assess the MSCs effect on inflammation, extracellular matrix remodeling and angiogenesis, in radiation-induced anorectal and colon damage. Repeated administrations of MSCs controlled systemic and local inflammation, normalized plasma CRP concentration, reduced *in situ* in both expression of inflammatory cytokines, increase in macrophage recruitment and augmented Foxp3 expression in rectal mucosa. MSC injections reversed radiation-induced fibrosis by reducing collagen deposition and expression of Col1a2/Col3a1 and TGF-β/CTGF, and by modifying the MMP/TIMP balance. In conclusion, in a pig model of proctitis, repeated injections of autologous MSCs effectively reduced inflammation and fibrosis and are a promising therapy for radiation-induced rectal damage.

Poster Board Number: F-3350

EFFECT OF *IN VITRO* CULTURE CONDITION AND IFN-Γ PRECONDITIONING ON GENE EXPRESSION PROFILE OF MESENCHYMAL STEM CELLS FOR IMMUNOSUPPRESSION

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Mesenchymal stem cells (MSCs) have been of particular interest due to their immunosuppressive capacities; however, few clinical trials revealed satisfactory results despite a large number of successfully demonstrated pre-clinical data. In this study, the effects of the cell density when MSCs were harvested and presence of IFN-γ

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pre-conditioning on the immunosuppressive properties of MSCs were investigated. The gene expression profiles obtained from microarray analysis were initially analyzed so that CXCR7, MUC1, PTGES, and ULBP1, known to be potentially involved in various immune responses, were found to be significantly up-regulated in MSCs harvested at a higher cell density. Especially PTGES and ULBP1 were further confirmed for their immunosuppressive activities so that MSCs with PTGES or ULBP1 siRNA were found to restore the T-cell proliferation. In addition, several factors such as CD274, HLA-DRA, INDO, CCL8, CXCL10, and CXCL9 were significantly up-regulated in IFN-γ pre-conditioned MSCs. It was then proved that MSCs treated with INDO shRNA recovered the T-cell proliferation rate in vitro, and the enhanced immunosuppressive properties of IFN-γ pre-conditioned MSCs were also demonstrated by the survival rates of in vivo GvHD models; the presences of MSCs expressing INDO were in fact detected in the mouse models. Also, in vivo GvHD models injected with IFN-γ treated MSCs possessing inhibited INDO expression showed reduced immunosuppressive functions so that INDO was particularly thought to be a key molecule involved in IFN-γ mediated immunosuppression of MSCs. Therefore, as the *ex vivo* expansion of MSCs has been considered as an essential factor, collecting better behaving MSCs as an effective immunomodulator may lead to the success of clinical trials by pre-conditioning IFN-γ on MSCs which have been previously cultured at a higher cell confluence.

Poster Board Number: F-3351

MESENCHYMAL STEM CELLS: A DOUBLE-EDGED SWORD IN REGULATING IMMUNE RESPONSES

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Mesenchymal stem cells (MSCs) have been employed successfully to treat various immune disorders in animal models and clinical settings. We have reported that MSCs can become highly immunosuppressive upon stimulation by inflammatory cytokines, an effect exerted through the concerted action of chemokines and nitric oxide (NO). We show here that MSCs could also enhance immune responses. This immune promoting effect occurred when proinflammatory cytokines are inadequate to elicit sufficient NO production. When iNOS production was inhibited or genetically ablated, MSCs strongly enhance T cell proliferation in vitro and DTH response *in vivo*. Furthermore, iNOS-/- MSCs could significantly inhibit melanoma growth. It is likely that in absence of NO, chemokines act to promote immune responses. Indeed, in CCR5-/-CXCR3-/- mice, iNOS-/- MSCs have reduced immune promoting effect. Thus, NO acts as a switch in MSC-mediated immunomodulation. More importantly, the dual effect on immune reactions was also observed in human MSCs, in which IDO acted as a switch. This study provides novel information for better understanding of the pathophysiological roles of stem cells.

Poster Board Number: F-3352

MI-RNA 150 AND 7 REGULATORS OF MSC ACTION ON COLON CANCER INFLAMMATORY TUMOR MICROENVIRONMENT.

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This study is the first step toward the use of MSC therapy to reverse pelvic radiotherapy complications on healthy tissue without secondary effect on residual cancer. A rat model of colorectal carcinogenesis close to human cancer was used as preclinical model. MSC treatment reduces cancer significantly lowering the number of adenomas and adeno-carcinomas and extends the life of animals. Anti-cancer effect of MSC was mediated by their immunologic properties. In adenocarcinoma of MSC-treated rats, monocytes/macrophages CD68+ infiltration was lowered and lymphocytes CD3+ increased. MSC induce macrophage to turn into regulatory cells involved in phagocytosis, inhibiting the production of pro-inflammatory cytokines. MSC decrease NK cells and rTh17 cell activities, Treg recruitment, CD8 + lymphocytes and endothelial cells number, restore Th17 cell activity. MiRNA. mi-150 and miRNA-7 are the key effectors. Mi-150 inhibits tumor invasion and mi-RNA-7 regulates negatively the pathway EGFR / AKT promoting cell death. MSC infusion have a durable action on colon cancer development by modulating the immune component of the tumor microenvironment For the first time, two mi-RNA were identified as responsible of MSC anti-cancer effect. This study is the first step toward use of MSC in therapy to alleviate the effects of radiation exposure in patients.

Poster Board Number: F-3353

EXPRESSION AND DELIVERY OF CARBOXYLESTERASES BY THERAPEUTIC NEURAL STEM CELLS: IMPLICATIONS FOR CLINICAL USE

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Carboxylesterases (CE) are enzymes found in a wide range of organisms, from humans to bacteria. Two major CEs exist in humans, human liver CE (hCE1; CES1) and human intestinal CE (hiCE; CES2). Selective expression of CE enzymes at the tumor site may be used for enzyme/prodrug tumor-targeted therapy. Neural stem cells (NSCs) have been investigated in preclinical models to deliver oncolytic viruses, pro-apoptotic proteins and enzymes that can catalyze the conversion of a pro-drug to an active therapeutic in disseminated or inaccessible tumor models (a Phase I clinical trial using NSCs to deliver cytosine deaminase enzyme to activate 5-FC to FU in patients with recurrent glioblastoma is underway at City of Hope). hCE1, an intracellular enzyme, was modified by site-directed mutagenesis by truncation of the C-terminus to allow for secretion, and substitution of 8 internal residues with those derived from the rabbit (r)CE sequence, which can better cleave the pro-drug, irinotecan, (CPT-11) to the active metabolite, SN-38, a potent topoisomerase I inhibitor. In this study, we tested the hypothesis that rCE and the engineered hCE1, termed hCE1m6, can be used in NSC-mediated anticancer therapy. We studied the use of HB1.F3.CD therapeutic NSCs, a well-characterized clonal cell line derived from human fetal telencephalon, for its potential to express and secrete rCE and hCE1m6. HB1.F3.CD cells were transduced by recombinant adenovirus encoding rCE or hCE1m6 genes. In vitro cell viability, growth and migration assays revealed that tumor-targeted migration of NSCs was not affected by expression of rCE or hCE1m6. NSCs secreted functional CE enzyme into the media, as monitored by a spectrophotometric assay using conversion of o-nitrophenyl acetate to o-nitrophenol. Exposure of human glioma cells to media derived from the HB1.F3.CD cells, transduced with rCE or hCE1m6, decreased the IC50 for tumor cell killing in the

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presence of CPT-11 by 100 to 1000-fold when compared to CPT-11 alone. The rCE and hCE1m6 enzymes were evaluated for immunogenicity to human peripheral blood mononuclear cells (PBMC) isolated from healthy volunteers using mixed lymphocyte reactions (MLR). hCE1m6 induced lower responses by PBMCs from 20 donors when compared with rCE in MLR assays. Using microdialysis assays, we also demonstrated that NSCs express functionally active rCE or hCE1m6 enzymes *in vivo* in rat brains and their expression catalyzes the conversion of CPT-11 to SN-38. Taken together, these data demonstrate the rationale for expression and delivery by NSCs of the engineered human CE, hCE1m6, to activate CPT-11 in a tumor targeting strategy.

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THE USE OF AUTOLOGOUS MESENCHYMAL STROMAL CELLS FOR TREATMENT OF NEURODEGENERATIVE DISORDERS - PAST ACHIEVEMENTS AND FUTURE GOALS

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Future treatment of multiple sclerosis (MS) aims at restoring myelination and neurological functions as well as re-induction of self-tolerance. We studied the role of mesenchymal stromal stem cells (MSC) in experimental autoimmune encephalitis (EAE) and found that treatment of mice with bone marrow derived MSCs resulted in significant suppression of anti-self reactivity and improved clinical and pathological disease manifestations. Our preliminary results of a phase I/II open clinical trial to evaluate the feasibility and safety of intrathecal and intravenous administration of autologous bone marrow derived MSCs in patients with severe MS failing conventional modalities suggested that treatment with MSCs is feasible, safe and potentially effective. No major side effect were developed or reported during a follow up period of nearly 4 years. Based on our successful preliminary pilot study in 13 patients with MS and 14 with ALS, a total of >150 patients were treated with autologous bone marrow derived MSCs, mostly >100 patients with MS alone were treated at our center with MSCs administered partially intrathecally (10^6 MSC/Kg) and additional 5 - 10x10^5/Kg intravenously. The treatment was uneventful with headache due to lumbar puncture being the most frequent complaint (70%) and self-limited subfebrile temperature in 25% and no serious side effect. A total of 60 % of patients reported improved outcome, in some with very significant objective improvement of disease manifestations with subjective and objective improvement of EDSS score. More recently, we have discovered a new proprietary technology which allows differentiation of bone marrow, adipose tissue derived and cord/placenta derived MSCs into different types of neural stem cells that in principle may be used for cell replacement therapy. These results suggest that treatment with MSCs and in the near future differentiated MSCs may become the treatment of choice for MS, also applicable for treatment of other neurodegenerative disorders.

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CHALLENGES AND BEGINNINGS OF STEM CELL RESEARCH IN MONGOLIA

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Mongolia, a country that is little known beyond its borders, has a nomadic heritage. Mongolia is developing at the fastest rate in its history, and the International Monetary Fund expects growth to average 14% a year within the next five years. Mongolia's transition from a communist country to a stable democracy is unique among Asian countries that were not part of the former Soviet Union. Prior to 1989, education and research in engineering and the sciences were developing strategically but lagging behind in high-level basic research and advances in technology. The Mongolian market economy and democratic political change have revealed opportunities for understanding and experiencing state-of-the-art technology and innovation that play major roles in advancement of development. Not only biomedicine but also stem cell research are new concepts in Mongolia and, in the absence of policies and institutions and research related to stem cells research, we have had to start our work from the ground up. Recent private and government cooperation in cord blood banking are, however, helping in the establishment of stem cell research in Mongolia. Cord blood is the most accessible source of stem cells and can be collected at the two main hospital-birth centers in the capital city, Ulaanbaatar, which has one and a half million residents, almost half of the entire population of Mongolia. At present, we are working on standardizing the preparation of stem cells from cord blood and on the storage and monitoring of such cells on a small to medium scale for stem cell banking, all of which are essential for future research. We plan to focus on the implications of allogeneic and autologous transplantation of stem cells from cord blood or of cord blood, itself, in a clinical setting. Our research will require a laboratory that meets international standards for stem cell research based on our cord blood banking units. It is a potential model, in Mongolia, for further stem cell research and application in larger facilities that will be established when there is more national and international involvement. Our goal, within the laboratory setting, is not only to establish private cord-blood banking but also to set up a research base, where we can monitor stem cells with respect to proliferative capacity and pluripotency. In a broader context, we shall continue to educate the Mongolian public and private sectors, as well as governing bodies, about the importance of stem cells for research, industry, the economy and public health. Current collaboration between the private sector and government agencies is moving us in the right direction. Our efforts will be greatly enhanced by contacts and collaborations with stem cell biologists in the international community, which will bridge the great distance between Mongolia and researchers in the advanced stem-cell laboratories and centers in the rest of the world.

Poster Board Number: F-3356

ANTIGRAVITY-MUSCLE ATROPHY IN MICROGRAVITY AND MUSCLE REGENERATION BY STEM CELLS

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Prolonged exposure to microgravity causes muscular atrophy due to unloading. Significantly, severe atrophy occurs in the soleus mus-

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cle, which is essential in maintaining balance and posture. Therefore, we transplanted human umbilical cord-derived mesenchymal stem cells (UC-MSCs) into rats set up using the hindlimb suspension (HS) rodent model and then observed its efficacy. UC-MSCs (1.0 x 10^6 cells) labeled with fluorescent nanoparticle were transplanted into 3 weeks HS white Sprague Dawley (SD) rats to observe if any reduction in atrophy occurs. 16S rRNA RT-PCR was carried out on various organs to detect whether the transplanted cells move to parts other than the soleus muscle. Morphological analysis and lactate assay were performed to measure the muscle recovery. As a result, transplanted stem cells were only detectable in the soleus muscle. Also, reduction in lactate and recovery of muscle fibers (similar to normal) were observed in stem cell injected rats compared to the control. Immunofluorescent analys shows that it takes 1 week for the stem cells to spread over the muscle. These results demonstrated that the transplanted UC-MSCs significantly repaired muscle atrophy in simulated microgravity. Therefore, our results show that intramuscular transplantation of UC-MSCs can be used in the treatment of muscle atrophic diseases. Also, we believe further applications in the effective treatment of astronauts who undergoing space missions or athletes who were injured in sports fields.

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PERIODONTAL TISSUE ENGINEERING WITH COMBINATION OF ADIPOSE TISSUE-DERIVED STEM CELLS AND PLATELET RICH PLASMA

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Objectives: Periodontitis results in the loss of connective tissue and bone support and is a major cause of tooth loss in adults. The ultimate goal of periodontal therapy is to regenerate the periodontal tissues that are lost as a result of periodontitis. The purpose of this study is to examine the efficacy of the combination of adipose tissue-derived stem cells (ASCs) and Platelet-Rich Plasma (PRP) in a canine periodontal tissue defect model. Methods: ASCs were prepared from inguinal fat pads of beagle dogs (six month, weight 7-10 kg), and cultured in control medium (DMEM+10%FBS). The penetrated furcation defects were created in the mandibular premolars, 1.5 x 107 cells/ml ASCs with PRP, which prepared from autologous blood, were implanted into the experimental group (n=10). While no treatment and PRP independent were performed as the controls (n=20). After one and two months implantation, histologic examination and immunohistochemical staining for osteocalcin were performed. The new-formed bone area and new-formed cementum length were measured on the section of hematoxylin and eosin staining. Statistical analysis was performed by student t-test using the Microsoft Excel software. Results: After one-month implantation, although the statistics difference of new-formed bone was not seen between all of groups, osteocalcin positive cells onto dental root in the defect area was evident in ASCs with PRP group. Two months after implantation, cementum and periodontal ligament like structure were clearly observed in defect area of ASCs with PRP group, and these structures were connected with new-formed bone. Only ASCs and PRP group, statistics differences of new-formed bone and cementum were seen between one and two month implantation. Conclusions: These findings suggest that ASCs with PRP could contribute to periodontal tissue regeneration in the canine model. The combination of ASCs and PRP may be a useful in future clinical cell-based therapy for periodontal tissue engineering.

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