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Conference Program

Meeting room – CESAR 1,2,3 & 4

**Thursday 14th December**

Keynote Speaker
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Rudolf Jaenisch
Nuclear Cloning, Embryonic Stem Cells and Cell Therapy: Promise, Problems, Reality

19:00 Welcome reception and buffet at poolside
21:00 Entertainment

**Friday 15th December**

**Session 1: Stem Cells and Cancer**

**Chair:** Connie Eaves

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Stem cell self-renewal, cancer cell proliferation, and aging

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Connie Eaves
Exploring the Stem Cell Concept: from Blood to Breast

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Hans Clevers
Wnt and Notch cooperate to maintain proliferative compartments in crypts and intestinal neoplasia

11:30 - 11:45
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Rebecca Morris
Evidence that hair follicle stem cells are carcinogen target cells

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Irv Weissman
No title available

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Snorkelling
Tulum visit

17.15 – 17.45
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Interrogating development and disease using nuclear transfer and stem cells

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Promiscuous global transcription in pluripotent embryonic stem cells

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The Transcriptional Program of Pluripotency

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Marica Grskovic
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Gregory Korbutt
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Drinks break in hotel lobby

**Session 3: Differentiation potential of adult stem cells**
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Kyung-Sun Kang
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Francisco Silva
Reprogramming of Post-Natal Male Germ Cells

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Simon Smukler
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Freda Miller
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Lunch on Patio Mexicano and free time

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Nobuyasu Maki
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René Ketting
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Carlos Pereira
Chromatin events underlying lymphocyte reprogramming to pluripotency

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Laura O’Neill
Comparison of histone modifications on key regulatory genes during early mouse development using C-ChIP

10.00 – 10.30
Brian Hendrich
Epigenetic silencing and cell fate decisions in pluripotent cells

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Wolf Reik
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09.30 – 09.45
Carlos Pereira
Chromatin events underlying lymphocyte reprogramming to pluripotency

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Abstracts – Oral

Rudolf Jaenisch

Abstract 1

Nuclear Cloning, Embryonic Stem Cells and Cell Therapy: Promise, Problems, Reality

Rudolf Jaenisch

Whitehead Institute for Biomedical Research and Department of Biology, MIT, Cambridge, MA 02124, USA

The full term development of sheep, cows, goats, pigs and mice has been achieved through the transfer of somatic cell nuclei into enucleated oocytes. Despite these successes, mammalian cloning remains an inefficient process, with a preponderance of reconstructed embryos failing at early to midgestation stages of development. The small fraction of conceptuses that survive to term are characterized by a high mortality rate and frequently display grossly increased placental and birth weights. It is likely that inappropriate expression of crucial developmental genes may contribute to lethality of cloned embryos.

One of the key issues raised by nuclear cloning is the question of genomic reprogramming, i.e. the mechanism of resetting the epigenetic modifications that are characteristics of the adult donor nucleus to ones that are appropriate for an embryonic cell. Also, a major unresolved question that was raised half a century ago in the seminal nuclear transfer experiments with amphibians is whether nuclei of terminally differentiated cells can be reprogrammed to direct development of a new organism. To address these questions we have used ES cells, terminally differentiated lymphoid cells, neurons and cancer cells as donors for nuclear transplantation. Our results show that the efficiency of generating cloned mice from an embryonic donor cell nucleus is significantly higher than cloning from a terminally differentiated donor cell nucleus. This is consistent with the notion that the differentiation state of the donor nucleus profoundly affects the efficiency of epigenetic reprogramming after nuclear transfer into the egg. Indeed, expression analyses indicated that the failure to properly activate key embryonic genes that are constitutively expressed in the embryonic but not in the adult donor cell represents one of the most important causes for early clone demise. Likely this is due to the genome of an adult cell being less amenable to the reprogramming activity of the oocyte than that of an embryonic cell. It is crucial to understand the molecular circuitry in a stem cell and that in a differentiated somatic cell. I will discuss our progress in understanding the key regulatory mechanisms that are crucial for self-renewal of stem cells and our approach in using such information to understand and possibly improve the low efficiency of genomic reprogramming after nuclear transplantation.

An emerging consensus is that somatic cell nuclear transfer (SCNT) for the purpose of creating a child (also called “reproductive cloning”) is not acceptable for both moral and scientific reasons. In contrast, SCNT with the goal of generating an embryonic stem cell line (“therapeutic cloning”) remains a controversial issue. Although therapeutic cloning holds the promise of yielding new ways of treating a number of degenerative diseases, it is not acceptable to many because the derivation of an embryonic stem cell line from the cloned embryo (an essential step in this process) necessarily involves the loss of an embryo and hence the destruction of potential human life. I will describe a proof of principle experiment of the “Altered Nuclear Transfer” (ANT) approach, a modification of SCNT that has been suggested by Hurlbut. The purpose of ANT is to avoid the generation of embryos that have any potential for fetal development but still can be the source of “customized” ES cells for cell therapy. The intention of this experiment has been to provide a basis for a more rational discussion of these complex issues.
Sean Morrison

Abstract 2

Stem cell self-renewal, cancer cell proliferation, and aging

Sean J. Morrison

Howard Hughes Medical Institute,/ University of Michigan Center for Stem Cell Biology, Ann Arbor, MI, USA

Networks of proto-oncogenes and tumor suppressors that control cancer cell proliferation also regulate stem cell self-renewal and stem cell aging. Proto-oncogenes promote regenerative capacity by promoting stem cell function but must be balanced with tumor suppressor activity to avoid neoplastic proliferation. Conversely, tumor suppressors inhibit regenerative capacity by inhibiting proliferation, promoting cell death or promoting senescence in stem cells. For example, the polycomb family proto-oncogene, Bmi-1, is consistently required for the self-renewal of diverse adult stem cells, as well as for the proliferation of cancer cells in the same tissues. Bmi-1 promotes neural stem cell self-renewal partly by repressing the expression of Ink4a and Arf, tumor suppressor genes that are commonly deleted in cancer. Despite ongoing Bmi-1 expression, Ink4a expression increases with age, reducing stem cell frequency and function. Increased tumor suppressor activity during aging therefore partly accounts for age-related declines in stem cell function. Thus networks of proto-oncogenes and tumor suppressors have evolved to coordinate regenerative capacity throughout life. Imbalances within such networks cause cancer or pre-mature declines in stem cell activity that resemble accelerated aging.
Charles Vinson

Abstract 3

AP-1 activity regulates epithelial cell identity

Michael J. Gerdes, Maxim Myakishev, Nicholas A. Frost, Stuart H. Yuspa, Charles Vinson

National Cancer Institute, Center for Cancer Research, Bethesda, MD 20892

To examine the consequences of inhibiting AP-1 transcription factors in skin, transgenic mice were generated that use the tetracycline system to conditionally express A-FOS, a dominant negative that inhibits AP-1 DNA binding. Older mice develop mild alopecia and hyperplasia of sebaceous glands, particularly around the eyes. When A-FOS was expressed during chemical-induced skin carcinogenesis, mice do not develop characteristic benign and malignant squamous lesions, but instead develop benign sebaceous adenomas containing a signature mutation in the H-ras proto-oncogene. Inhibiting AP-1 activity after tumor formation caused squamous tumors to transdifferentiate into sebaceous tumors. Furthermore, re-activating AP-1 in sebaceous tumors results in a reciprocal transdifferentiation into squamous tumors. In both cases of transdifferentiation, individual cells express molecular markers for both cell types indicating individual tumor cells have the capacity to express multiple lineages. Molecular characterization of cultured keratinocytes and tumor material indicates that AP-1 regulates the balance between the wnt/β-catenin and hedgehog signaling pathways that determine squamous and sebaceous lineages respectively. ChIP analysis indicates that c-Jun binds several wnt promoters, which are misregulated by A-FOS expression suggesting that members of the wnt pathway can be a primary targets of AP-1 transcriptional regulation. Thus AP-1 activity regulates tumor cell lineage and is essential to maintain the squamous tumor cell identity.
Forced expression of PDGF-B in astrocytes and glial precursors induces brain tumors in p53 null mice

Hede S.M. 1, Hansson I. 2, Afink G.B. 1,2, Eriksson A. 1, Nazarenko I. 1, Andrae J. 1,2, Westermark B. 2 and Nister M. 1,2

1 Department of Oncology-Pathology, Karolinska Institutet, CCK R8:05, SE-17176, Stockholm, Sweden
2 Department of Genetics and Pathology, Uppsala University, SE-75185, Uppsala, Sweden

Glioblastoma is the most common and malignant type of human brain tumors. Primary glioblastomas that develop de novo are characterized by amplification and over expression of EGFR and loss of heterozygozity (LOH) on chromosome 10. Secondary glioblastomas on the other hand are believed to have developed from lower grade gliomas and frequently have mutations of p53 and LOH on chromosome 17p. The importance of p53 in turnover of neural stem cells and progenitor cells in the SVZ has recently been investigated in p53 null mice. It is well known that the growth factor PDGF (platelet derived growth factor) and its tyrosine kinase receptors are expressed in human brain tumors. In order to investigate the role of PDGF in brain tumor formation we have generated a transgenic mouse model where human PDGF-B is expressed in the astrocytes and glial precursor cells using the human GFAP promoter. Two GFAP-PDGFB transgenic lines were generated, crossed with p53 null mice and followed for up to six months. Approximately 60 percent of the GFAP-PDGFB/p53 null mice developed brain tumors, while the control groups with the PDGFB transgene alone, wild type mice or p53 null mice developed no brain tumors. The tumors show many similarities with human glioblastomas. They are diffusely growing and invasive, have microvascular proliferations as well as necrotic areas. Some spread along the meninges surrounding the brain and infiltrating microglial cells are seen in most of the tumors. Others spread out on the ventricular lining. The brain tumors are usually undifferentiated, expressing cellular markers such as nestin and vimentin, but also expressing GFAP and Tuj1. In situ hybridization for PDGFR- and - show that PDGFR- is strongly expressed in all tumor cells, suggesting an autocrine loop driving the tumor growth.
Connie Eaves

Abstract 5

Exploring the Stem Cell Concept: from Blood to Breast

C. Eaves, B. Dykstra, M. Bowie, D. Kent, P. Eirew, A. Raouf and J. Stingl

Terry Fox Laboratory, BC Cancer Agency and University of British Columbia, Vancouver, Canada

Much interest is currently focused on the concept that the lifelong functional integrity of many normal adult tissues is sustained by a rare subset of undifferentiated but tissue-restricted stem cells. One approach to the identification of such cells has been to demonstrate their exclusive presence within a defined subpopulation of cells that can be isolated from the tissue of interest. This, in turn, requires the establishment of suitably robust and quantitative assays that specifically detect the developmental attributes of a stem cell of that tissue. Such assays were first devised for the hematopoietic system and more recently have been created for the mammary gland. The fact that the same principles of sustained and complete tissue regeneration by clonal populations to detect stem cell activity and the use of limiting dilution principles to quantify the frequency of such cells could be readily transferred from the hematopoietic system to the mammary gland suggests that similar strategies will prove useful for identifying and characterizing other types of tissue stem cells. With the advent of methods to obtain and investigate highly purified hematopoietic stem cells at the single cell level, evidence of unanticipated, preprogrammed heterogeneity in the differentiation properties of these cells has been obtained and a novel checkpoint in their development has been identified. Initial studies of mammary stem cells suggest strong parallels with hematopoiesis but also highlight evidence against quiescence or dye efflux properties being a general feature of stem cells. These findings challenge the simplicity of current stem cell concepts and invite new definitions of developmental potential based on more precise descriptions of cellular epigenomes.
Hans Clevers

Abstract 6

Wnt and Notch cooperate to maintain proliferative compartments in crypts and intestinal neoplasia

Hans Clevers

Hubrecht Laboratory, Netherlands Institute for Developmental Biology of the Royal Netherlands Academy of Arts and Sciences

Mutations in the Wnt pathway components APC, beta-catenin and conductin all induce sustained complex formation of the co-activator beta-catenin with TCF transcription factors. The resulting transactivation of TCF target genes is believed to represent the primary transforming event in colorectal cancer (CRC). Yet, the consequence of the presence of mutationally activated beta-catenin/TCF in fully transformed CRC cells is unknown. We have constructed CRC cell lines carrying inducible dominant-negative TCF constructs. Inhibition of beta-catenin/TCF resulted in a rapid G1 arrest. DNA array analysis revealed the downregulation of a small set of transcripts. These genes were expressed in polyps, but also, physiologically, in the crypt progenitor compartments of the colon. By contrast, we observed the induction of multiple marker genes of intestinal differentiation upon inhibiting beta-catenin/TCF in CRC cells. We conclude that beta-catenin/TCF inhibits differentiation and imposes a crypt progenitor phenotype on CRC cells. Moreover, inhibition of beta-catenin/TCF activity restores the differentiation program, despite the presence of multiple other mutations in CRC.

TCF target gene expression is always restricted to the crypt, but target genes can be subclassified based on expression patterns within the crypt. We have tentatively identified at least three target genes which are expressed uniquely in the crypt stem cells. The Wnt cascade is not the only signaling pathway controlling cell fate along the crypt-villus axis. Upon conditional removal of the common Notch pathway transcription factor CSL/RBP-J2, we observe a rapid, massive conversion of proliferative crypt cells into post-mitotic goblet cells. A similar phenotype was obtained by blocking the Notch cascade using a gamma-secretase inhibitor. The inhibitor also induced goblet cell differentiation in intestinal adenomas. Our data imply that gamma-secretase inhibitors, developed for Alzheimer disease, may be of therapeutic benefit in colorectal neoplastic disease.

Taken together, maintenance of undifferentiated, proliferative cells in crypts and adenomas requires the concerted activation of the Notch and Wnt cascades.
Benign and malignant cutaneous neoplasms can be induced in mice following a subthreshold exposure to a carcinogen (initiation) and subsequent chronic regenerative epidermal hyperplasia (promotion). We investigated the cellular origins of mouse skin tumors using Krt1-15CrePr1;R26R transgenic mice which express an RU486 inducible Cre in the stem cells of the hair follicle bulge. To study the origins of skin tumor development, mice were initiated with a single topical application of the carcinogen, 7, 12-dimethylbenz[a]anthracene (DMBA) followed one week later by application of RU486 and subsequent tumor promotion with 12-O-tetradecanoylphorbol-13-acetate (TPA). Immediately after induction of the recombinase, only hair follicle bulge cells stained blue with X-gal histochemistry as we previously reported. During tumor promotion, blue cells were found first in squamous hyperplastic foci, and with continued tumor promotion, in papillomas and carcinomas. The blue cells in the lesions included proliferative cells as well as stratified and terminally differentiated cells. Bulge stem cells contributed to over 40% of the lesions studied thus far. Estimated percentages of blue cells in the center of the lesions ranged from about 1% to about 70%. Analysis of blue and non-blue tumor-involved cells disclosed the signature codon 61 mutation in the Ha-ras gene in the tumors but not in uninvolved hyperplastic epidermis or hair follicles. We conclude from these results that stem cells in the hair follicle bulge are skin tumor initiating cells. The cellular origins of the non-blue tumor cells are currently under investigation. This work was supported by CA97780 from the National Cancer Institute to RJM and GC.
Irving Weissman

Abstract 8

No abstract available at time of print
Kevin Eggan

Abstract 9

Interrogating development and disease using nuclear transfer and stem cells.

Kevin Eggan

The Stowers Medical Institute, The Harvard Stem Cell Institute and The Department of Molecular and Cellular Biology, Harvard University

Advances in cloning by nuclear transfer (NT) and ES cell/somatic cell hybridization have demonstrated that the mammalian oocyte and embryonic stem cells carry activities that can relieve the constraints imposed by cellular differentiation and return the nucleus of an adult cell to a totipotent embryonic state, a process termed epigenetic reprogramming. It has been our primary interest to understand the mechanisms by which reprogramming occur.

In particular, we are determining the nature of epigenetic information that is and is not reprogrammed (i.e., aspects of DNA methylation and chromatin structure), the times at which reprogramming occurs and the identities of the molecular machinery that accomplish reprogramming. In addition, we are using NT and a variety of other approaches to develop human embryonic stem (ES) cell lines that carry the genes that are responsible for human neurodegenerative diseases. It is our hope that these cell lines could be used both as in vitro models for the study of these diseases and as potential sources of material for cell replacement therapy.
Eran Meshorer

Abstract 10

Promiscuous global transcription in pluripotent embryonic stem cells

Eran Meshorer, Tom Misteli

National Cancer Institute, NIH, Building 41, Room B508, 41 Library Dr., Bethesda MD 20892

Embryonic stem (ES) cells, derived from the inner cell mass of the blastocyst, are unique in their ability to both self-renew and differentiate into the three germ layers, endoderm, ectoderm and mesoderm. Pluripotent ES cells display distinct chromatin features, characterized by global changes in chromatin organization, higher H3 and H4 histone acetylation, reduced H3 lysine 9 methylation and hypermobile fractions of chromatin associated proteins, including core histones. These features suggest that ES cells might be globally transcriptionally more active than differentiated cells. Using a combination of imaging techniques, molecular tools and global tiling microarray analysis, we characterized the transcriptional landscape of differentiating mouse ES cells into neuronal progenitor cells. We further show that undifferentiated mouse ES cells have a de-condensed chromatin structure; low-level expression of tissue and lineage specific transcripts; relatively high levels of expression of repetitive sequences and transposable elements; higher global transcriptional activity, and elevated levels of activity-associated chromatin modifications and proteins implicated in chromatin remodeling. Taken together, these results suggest that undifferentiated ES cells have a globally open chromatin structure maintained by chromatin remodeling proteins and as a consequence, the de-condensed chromatin gives rise to promiscuous transcription leading to low level expression of lineage-specific genes and repetitive sequences. We propose that promiscuous transcription is an important hallmark of pluripotency and lineage specification and that differentiation promotes gene silencing by chromatin condensation and heterochromatin formation.
Marica Grskovic

Abstract 11

Systematic Identification of Novel Regulatory Sequences and Transcriptional Regulators in Mammalian Pluripotent Cells

Marica Grskovic, Christina Chaivorapol, Alexandre Gaspar Maia, Hao Li, Miguel Ramalho-Santos

University of California, San Francisco; CA 94143, USA

One of the defining features of embryonic stem (ES) cells is their ability to differentiate into all germ layers of the organism - an attribute that we define as pluripotency. Understanding the transcriptional regulation of pluripotency is of fundamental interest and will greatly inform efforts aimed at directing differentiation of ES cells or reprogramming of somatic cells. We used a combination of gene expression profiling with computational genomic analyses and biochemical assays to systematically identify novel cis-regulatory sequences that control gene expression in pluripotent stem cells. We first analyzed the transcriptional profiles of mouse ES cells and Primordial Germ Cells (PGCs), the pluripotent cells of the germline, and identified clusters of genes up-regulated in pluripotent cells. These genes are enriched for roles in transcription, chromatin remodeling, cell cycle and DNA repair. We developed a novel computational algorithm, CompMoby, to systematically predict short DNA motifs that regulate gene expression. One of the predicted motifs corresponds to the functional Sox2/Oct-4 binding site in the Nanog promoter, validating our approach. We show that the novel motifs are preferentially active in undifferentiated mouse ES and germ cells, are sequence-specific and are required in the context of an endogenous promoter. Importantly, the activity of the motifs is also conserved in human ES cells. We hypothesize that these motifs recruit novel transcriptional regulators of ES cells. Indeed, we have identified several transcriptional factors that specifically bind to the identified regulatory motifs. One such factor is NF-Y, which has not previously been implicated in regulation of ES cells. Using RNAi interference, we demonstrate that NF-Y is strictly required for self-renewal of ES cells. We will discuss data on other proteins that bind the motifs, and present an integrated model of transcriptional regulation in pluripotent cells.

Our results provide novel insights into the transcriptional regulatory networks that underlie early mammalian embryogenesis and stem cell pluripotency, and offer new paradigms for manipulation of the differentiation potential of stem cells and reprogramming of somatic cells towards pluripotency.
Miguel Ramalho-Santos

Abstract 12

The Transcriptional Program of Pluripotency

Miguel Ramalho-Santos¹, Marica Grskovic¹, Alexandre Gaspar-Maia¹, Clara Chiu¹, Grace Wei¹, Connie Wong⁴, Christopher DeJonge², Ru-Fang Yeh², Meri T Firpo³, Matthias Hebrok¹, Renee A Reijo Pera⁴

¹ Institute for Regeneration Medicine and Diabetes Center, University of California, San Francisco
² Division of Biostatistics, University of California, San Francisco
³ Stem Cell Institute, Department of Medicine, University of Minnesota, Minneapolis
⁴ Human Embryonic Stem Cell Center and Center for Reproductive Sciences, University of California, San Francisco

Correspondence should be addressed to:
Miguel Ramalho-Santos, mrsantos@diabetes.ucsf.edu, +1 415 502-8543

Pluripotent stem cells, such as Embryonic Stem (ES) cells, can be propagated indefinitely in vitro and can give rise to all cell types in the body. My lab aims to understand the transcriptional regulation of pluripotent stem cells. We characterized the transcriptional profiles of mouse ES cells and other pluripotent stem cells, using Affymetrix microarrays. Importantly, our analysis included the in vivo counterparts of pluripotent stem cells, i.e., the inner cell mass of the blastocyst (ICM, source of ES cells) and Primordial Germ Cells (PGCs, source of Embryonic Germ Cells, which are also pluripotent stem cells). Furthermore, we also analyzed the transcriptional profiles of human ES cells and ICMs, and compared those to the mouse data. This comprehensive study provides great insight into the transcriptional program of pluripotent stem cells. The main conclusions of this work are:

i) all pluripotent cells express a common set of genes that may define pluripotency;
ii) ES cells are transcriptionally more similar to PGCs than to the ICM;
iii) all the stages of PGCs analyzed (9.5-13.5 dpc), the most similar to ES cells is the 11.5 dpc (mid-gestation) stage;
iv) the similarities between ES cells and 11.5 dpc PGCs are due to an enrichment in chromatin remodeling and RNA processing activities.

Our data are thoroughly validated by quantitative RT-PCR. Our results show that ES cells are not the in vitro equivalent of the ICM, as generally assumed, but rather have high similarities to germ cells. These results have profound implications regarding the origin, regulation and practical applications of ES cells.

With these data in hand, we began to dissect the transcriptional program of pluripotency. We focused on three main approaches:

i) the bioinformatic identification and biochemical validation of transcription factor binding sites responsible for gene expression in ES cells;
ii) an RNAi screen to identify novel factors required for ES cell self-renewal;
iii) gene over-expression in somatic cells to explore the mechanisms of reprogramming to pluripotency.

I will discuss the main conclusions of these approaches. The data provide a comprehensive view of the transcriptional program of pluripotency.
Embryonic stem cells represent a theoretically inexhaustible source of precursor cells that can be differentiated into specific cell lineages. As with conventional organ transplant therapies, transplantation of ES cell products will be hindered by immunologic barriers. Genetically matched pluripotent embryonic stem cells generated via nuclear transfer (ntES cells), or parthenogenesis (pES cells), are a possible source of histocompatible cells and tissues. We have shown, in a proof of principle experiment, that customized embryonic cells generated via somatic cell nuclear transfer can be used to repair a genetic immunodeficiency disorder in mice (Rideout et al., Cell 2002). Generation of ntES cells facilitates performing gene correction and results in delivery of “autologous” tissues. However, the process remains inefficient, and to date has not been successfully practiced with human cells. Production of ES cells with defined histocompatibility loci can be generated at much higher efficiency by direct parthenogenetic activation of the unfertilized oocyte. Subsequently, cell lines can be genotyped and selected for MHC identity to the oocyte donor or specific individuals. Parthenogenetic murine ES cells display similar in vitro hematopoietic activity compared to regular ES cell lines, and blood derivatives can repopulate hematopoiesis in irradiated adult mouse recipients. These experiments establish murine models for generating histocompatible ES cell-derived tissue products.
Ron McKay

Abstract 14

Stem cell signaling pathways in acute and chronic neurodegenerative disease

Ron McKay

LMB/NINDS, NIH, Bethesda, MD

This presentation will focus on survival signaling mechanisms controlling (1) stem cell renewal in the ischemic brain and (2) maintenance of functional dopamine neurons in the adult.

Kinase cascades that interpret information delivered to the plasma membrane are essential mediators of the computations controlling cell numbers and identities in the developing brain but at present we have a poor understanding of the role of these pathways. Data will be presented showing that the Notch receptor activates the PDK1/Akt kinase pathway controls stem cell survival in vitro and promotes expansion of the stem cell compartment in the ischemic adult nervous system. Because midbrain dopamine neurons project axons that modulate synaptic activity across the entire forebrain their function is of great interest in neuroscience and in medicine. We have demonstrated that midbrain dopamine neurons are derived from the floor plate. The forkhead factor, foxa2, is required for the development of dopamine neurons and continues to be specifically expressed in adult dopamine neurons. Mice carrying only one copy of this gene show asymmetric behavioral abnormalities as they age and a corresponding late-onset asymmetric deficit in the substantia nigra neurons most affected in patients with Parkinsons disease. Foxa transcription factors are closely related to the foxo genes that have a central role in the cell survival we show is activated by the Notch receptor. The routine access allowed by cultured stem cells combined with powerful animal models promises new insight into cell signaling pathways controlling neuronal loss in ischemia and Parkinsons disease.
Differentiation of human embryonic stem cells into hepatic and pancreatic endoderm

Neta Lavon**, Ofra Yanuka*, Nissim Benvenisty*

*Department of Genetics, The Institute of Life Sciences, The Hebrew University, Jerusalem 91904, Israel
**Cedars-Sinai International Stem Cell Institute, Cedars-Sinai Medical Center, 110 George burns Road, Los Angeles CA 90048

Human embryonic stem cells (HESCs) are pluripotent cells that may serve as a source of cells for transplantation medicine, and as a tool to study human embryogenesis. Using genetic manipulation methodologies, we have investigated the potential of HESCs to differentiate into the various pancreatic cell types. We initially created various HESCs carrying the enhanced green fluorescent protein (eGFP) reporter gene under the control of either the insulin promoter or the pancreatic and duodenal homeobox factor-1, Pdx1, promoter. Our analysis revealed that during the differentiation of HESCs into embryoid bodies (EBs), we could detect green fluorescent cells when eGFP is regulated by Pdx1 promoter but not by insulin promoter. In order to examine whether we can induce differentiation into pancreatic cells, we have established human embryonic stem cell lines that constitutively express either Pdx1 or the endodermal transcription factor, Foxa2. Following differentiation into EBs the constitutive expression of Pdx1 enhanced the differentiation of HESCs towards pancreatic endocrine and exocrine cell types. Thus, we have demonstrated expression of several transcription factors that are downstream to Pdx1 and various molecular markers for the different pancreatic cell types. However, the expression of the insulin gene could be demonstrated only when the cells differentiated in vivo into teratomas. We conclude that although over-expression of Pdx1 enhanced expression of pancreatic enriched genes, induction of insulin expression may require additional signals that are only present in vivo.
Human embryonic stem cell derived pancreatic endocrine islet cells

Gregory S. Korbutt, Alana Eshpeter, Ray Rajotte, Jianjie Jiang, Melinda Au, Anish S. Majumdar

University of Alberta, Edmonton, Canada, and Geron Corporation, Menlo Park, CA, USA.

Human embryonic stem cells (hES) have been proposed as a potential unlimited source of tissue for transplantation in the treatment of Type 1 diabetes. A great deal of research has been conducted on the differentiation of mouse embryonic stem cells into insulin producing cells, much of which is controversial due to the addition of insulin to culture media. There has been new success in the field with a successful differentiation of hES into definitive endoderm. Recently, we have developed a defined protocol to differentiate human embryonic stem cells (hES) into cellular aggregates that are partially composed of pancreatic islet endocrine cells. We have further characterized these islet-like clusters (ILCs), and found them to be immunoreactive for islet markers such as insulin, C-peptide, glucagon, somatostatin, prohormone convertase 1/3 and 2, and PDX-1. We have observed that ILCs contain detectable insulin secretion by responding to a high glucose challenge in vitro. We have also transplanted hES derived ILCs under the kidney capsule of streptozotocin induced diabetic immuno-incompetant mice, and although blood glucose levels were not normalized, human C-peptide was detected in the serum of these mice. One explanation for not achieving normoglycemia in these mice is that a sub-optimal number of insulin-positive beta cells were transplanted. Nonetheless, the expression of islet endocrine cell markers by immunohistochemistry and RT-PCR (insulin, glucagon, PDX-1) was also maintained even after 70 days in vivo. These results show that ILCs differentiated from hES appear to be immature cells of pancreatic endocrine lineage, and it is possible that further purification, differentiation, and maturation of these cells could lead to the generation of a sufficient source of islets for transplant in patients with Type 1 diabetes.
Hiram Chipperfield

Abstract 17

The directed differentiation of human embryonic stem cells into insulin secreting beta cells


11 Biopolis Way, #05-06 Helios, Singapore 138667

The directed differentiation of hESC into insulin-secreting beta cells holds the promise of a therapy for type I diabetes. We found that differentiating hESC as embryoid bodies in an extra-cellular matrix combined with Activin A and BMP4 leads to the robust expression of Pdx1, a pancreas specific gene. Compared to untreated control cultures, this protocol resulted in the up-regulation of definitive endoderm markers such as Sox17 and FoxA2 in the first 10 days. This was followed by the induction of Pdx1 which peaks at around day 20, when up to 19% of the cells are Pdx1 immunoreactive. In some cases the Pdx1-positive progenitors formed complex branching structures reminiscent of the embryonic pancreas. Upon further differentiation the expression of mature beta cell markers such as Isl1, insulin, glucagon and somatostatin was up-regulated. Mature beta-like cultures secreted C-peptide into the media reaching a concentration of up to 20 ng/ml after two days. When these cells were implanted into mice, human C-peptide was found in the blood and urine. Analysis of recovered grafts indicated that the differentiated cell population retains pancreatic marker expression after transplantation into an animal. We are currently improving the efficiency of this differentiation protocol and moving towards clinical compliance with the expectation of conducting clinical trials for type I diabetes.
Stem cells are essential in many adult tissues for both homeostatic maintenance and regenerative replacement of differentiated cells. Different organs contain different stem cells, each of which produces a specific subset of the body’s cells. We have studied two distinct tissue-specific stem cells: hematopoietic (blood-forming) stem cells and myogenic (muscle-forming) stem cells as paradigms of adult stem cell function. In the hematopoietic system, this work has defined both intrinsic and extrinsic factors that cooperate to determine stem cell fate, and identified a transcriptional regulatory pathway that appears to coordinately control stem cell localization and proliferation. In the skeletal muscle, we defined phenotypic markers allowing the direct isolation of self-renewing myogenic stem cells, and have used these cells to investigate the requirements for muscle regeneration from transplanted stem cells and to begin to construct a myogenic lineage map that will enable a more refined molecular analysis of muscle cell fate decisions. In addition, using mouse models of muscular dystrophy and age-associated muscle degenerative disease, we have identified defects in stem cell maintenance and function that associate with impairment of muscle regeneration. Together, these studies shed light on both common and divergent mechanisms for controlling adult stem cell function and suggest novel approaches to modulate stem cell activity in the context of therapeutic tissue repair.
Kyung-Sun Kang

Abstract 19

Umbilical Cord Blood-derived Multi-potent Stem Cells for Cardiovascular Diseases

Young-Gun Lee, Sun Bo, Yong-Soon Lee, Kyung-Sun Kang

Adult Stem Cell Research Center, Laboratory of Stem Cell and Tumor Biology, Department of Veterinary Public Health, College of Veterinary Medicine, Seoul National University, Seoul 151-742, Korea

Buergers disease, also known as thromboangiitis obliterans, is a nonatherosclerotic, inflammatory, vasooclusive disease. It is characterized pathologically as a panangiitis of medium and small blood vessels including both arteries and adjacent veins, especially the distal extremities, the feet and the hands. There is no curative medication or surgery for this disease. In the present studies, we transplanted human leukocyte antigen (HLA)-matched human umbilical cord blood (UCB)-derived mesenchymal stem cells (MSCs) into 4 men with Buergers disease who had already received medical treatment and surgical therapies. After the stem cell transplantation, ischemic rest pain suddenly disappeared from their affected extremities. The necrotic skin lesions were healed within 4 weeks. In the follow-up angiography, digital capillaries were increased in number and size. In addition, vascular resistance in the affected extremities, compared with the preoperative examination, was markedly decreased due to improvement of the peripheral circulation. Because an animal model of Buergers disease is absent and also in order to understand human results, we transplanted human UCB-derived MSCs to athymic nude mice with hindlimb ischemia by femoral artery ligation. Up to 60% of the hindlimbs were salvaged in the femoral artery ligated animals. By situ hybridization, the human UCB-derived MSC was detected in the arterial walls of the ischemic hindlimb in the treated group. Therefore, it is suggested that human UCB-derived MSCs transplantation may be a new and useful therapeutic armament for Buergers disease and other similar ischemic or cardiovascular diseases.
Francisco Silva

Abstract 20

Reprogramming of Post-Natal Male Germ Cells

Francisco Silva

213 Technology Drive, Suite 100, Irvine, CA 92618

The pluripotent nature of embryonic stem cells (ESC) has created an increasing demand to translate the therapeutic potential of these cells to a clinical application. However, the clinical application of these cells is hindered not only by ethical hurdles, but by their ability to form teratomas and immune rejection upon transplantation. Like ESCs, embryonic germ cells (EGC) have been derived via culture induced reprogramming, however propagation and use of human fetal material has prevented the therapeutic development of EGCs. Recently, studies have demonstrated that the elasticity of the germ-line to undergo culture induced reprogramming is not exclusive to the pre-natal environment but extends to the post-natal environment. One study reported the derivation of ES-like cells from neonatal mice that expressed pluripotent markers, contributed to chimeric cell populations when injected into blastocysts, and generated teratomas. Studies by us and a German laboratory have demonstrated that pluripotent cells can also be derived from adult mice, which demonstrate the efficacy of culture-induced reprogramming of the post pubescent germ-line. In this report, we used a transgenic mouse model expressing Oct-4-GFP as a reporter marker to track adult germ cell (AGC) derivation. We found that growth factor reprogramming is a dynamic process involving down and up-regulation of Oct-4 expression. Furthermore, our AGCs are similar, but not identical to ES-like cells because AGCs produced chimeric mice but did not form teratomas. In addition we report that adult human germ cells can also be reprogrammed to become AGCs that are characteristically similar to murine AGCs, including Oct-4 up-regulation, pluripotent marker expressions (Nanog, SSEA-4, TRA-1-60, Rex-1 and Alkaline phosphatase), and the ability to undergo spontaneous and induced differentiation into cardiomyocytes, neural cells, chondrocytes and adipocytes. Thus, therapeutically reprogrammed adult germ cells can become a novel pluripotent stem cell source for use in autologous cell based regenerative medicine.
Adult mammalian pancreas-derived multipotent Stem Cells

Simon Smukler, Raewyn Seaberg, Margot Arntfield, George Bikopoulos, Feihan Dai, Simon Lee, Jonathan Lakey, Michael Wheeler, Derek van der Kooy

University of Toronto

The search for putative precursor cells within the pancreas has been a focus of extensive research, largely due to their potential use in the generation of new beta cells for therapeutic cell replacement strategies in the treatment of diabetes. We have reported the identification of pancreas-derived multipotent precursor (PMP) cells isolated from the adult mouse pancreas that can clonally proliferate and differentiate to produce pancreatic and neural lineage cells. The intriguing capacity of these cells to generate progeny of distinct germ layers - endodermal pancreatic and ectodermal neural – raises many questions as to their in vivo identity and ontogenic developmental compartment. The neural crest (NC) is a developmental structure with broad contribution to various lineages. We investigated whether PMPs were NC-derived using a transgenic mouse with Wnt1-expressing cells and their progeny indelibly fluorescently labeled. Wnt1 expression is thought to be restricted to the NC in the periphery. FACS sorting of labeled cells showed that all PMPs were in the non-fluorescent fraction. These data, in addition to RT-PCR analysis which demonstrated the absence of NC gene expression in PMP spheres, strongly suggests that they are not NC-derived. The in vivo identity of the PMPs remains unknown, and it has been demonstrated that insulin-expressing cells retain the capacity to divide in a limited manner. We investigated whether PMPs may actually express insulin in vivo using transgenic mice expressing GFP under control of the insulin promoter. FACS sorting revealed that the vast majority of the PMPs were contained in the insulin-positive fraction. This suggests that while the majority of the native insulin-expressing cells are differentiated beta cells, a subset of them actually represent a precursor population with more extensive capabilities. Next, we sought to determine whether the human pancreas contained cells analogous to those we identified in mice. Using purified, dissociated human islets, we found that a rare population of highly proliferative cells was present. The primary colonies produced could be passaged to produce secondary colonies. RT-PCR analysis of the colonies showed that they expressed precursor genes of both the neural and pancreatic lineages (e.g. Nestin, Sox2, Mash-1, Pax6, PDX-1, HNF3β, HNF4, Gata-4), while expression of mesodermal genes (Brachyury and Gata-1) was not detected. Further, differentiation markers of both lineages were also present. Neuronal and glial cell types were detected by the expression of β3-tubulin and GFAP, while endocrine pancreatic cells types were revealed by expression of Insulin, Glucagon, and Somatostatin. Finally, the functionality of the newly generated beta cells was verified by insulin secretion studies. These data suggest that the adult mammalian pancreas contains a population of insulin+ multipotent stem cells, capable of contributing to the neural and pancreatic lineages.
Freda Miller

Abstract 22

Skin-derived precursors (SKPs): basic biology to therapeutic utility

Freda Miller

Hospital for Sick Children, University of Toronto, Toronto, Canada

We have previously isolated and characterized a multipotent precursor from adult rodent and human dermis that will self-renew in culture, and that is capable of generating both neural and mesodermal progeny (termed SKPs for SKin-derived Precursors) (Toma et al., Nat. Cell Biol., 2001; Toma et al., Stem Cells, 2005). This lecture will describe our recent work where we have asked first about the endogenous role of these adult precursors, and second, where we have attempted to define the therapeutic potential of isolated, expanded SKPs. In this regard, we have recently demonstrated that SKPs represent a neural crest-related precursor that is laid down in skin during embryogenesis, and that persists in lower numbers into adulthood within at least one defined niche, the dermal papillae of hair and whisker follicles (Fernandes et al., Nat. Cell Biol, 2004). Work will be described that supports the concept that SKPs can reconstitute this niche, and that they can function in many ways as multipotent dermal precursors. Moreover, we have recently shown that SKPs can generate at least one functional neural cell type in vivo, myelinating Schwann cells (McKenzie et al., J. Neurosci., 2005), and studies will be described that address the therapeutic utility of SKP-derived Schwann cells. Together our studies suggest that SKPs represent embryonic neural crest-related precursors that persist in adult dermis where they play an important ongoing role in morphogenesis and tissue turnover, and that human SKPs provide an expandable population of human neural crest precursors for a variety of therapeutic purposes.
Cheng-Ming Chuong

Abstract 23

Evo-devo of stem cells and strategies of regeneration

Cheng-Ming Chuong

University of Southern California, Los Angeles, chuong@pathfinder.usc.edu, http://www-hsc.usc.edu/~cmchuong

In lower animals, cells are less specialized and many cells in the adult organism have the pluripotential property useful for regeneration or reproduction (e.g., hydra). In higher animals, cells / tissues are differentiated in a more diverse way and organized more complexly. The cost of specialization is the limited source of stem cells in the adult. Animals use different strategy to provide stem cells when they need them. Some stem cells are generated upon injury through de-differentiation (e.g., amphibian limb). Here we compare the management of stem cells by two "professional" reproductive organs: hair and feathers, which go through regenerative cycles physiologically.

Feathers and hairs form when birds and mammals arise from reptiles independently, yet both evolve to form the follicular structure as a result of convergent evolution. In hair, hair follicle stem cells are located in the bulge area and cycling go thorough by destroy of the lower follicles. In the feather follicle, it go thorough different dynamic changes during molting cycle. On the same bird, there are bilaterally symmetric feathers and radially symmetric feathers. We show feather epidermal stem cells are configured as a ring and reside in the proximal follicle. The ring is horizontally placed in radially symmetric feathers, but tilted toward the anterior (rachis) side in bilaterally symmetric feathers. At the ramogenic plane of flight feathers, there are asymmetric molecular gradients. RCAS mediated misexpression of Wnt3a, β-catenin or their antagonists convert bilateral to radial symmetry or initiate a new rachis. Local perturbations with growth factor coated beads show Wnt3a can re-orient barbs and create a duplicated rachis. The gradient is determined by the dermal papilla.

Learning how nature use different strategy to manage stem cells can inspire us with new concepts and new approaches to engineer stem cells into organs we desire.
Oleg Tsinkalovsky

Abstract 24

Fishing for Stem Cells: Isolation of Hematopoietic Stem Cells in Zebrafish

Oleg Tsinkalovsky¹, Audun Osland Vik-Mo², Sara Ferreira³, Ole Didrik Laerum¹, Anders Fjose³.

¹Stem Cell Research Group, the Gade Institute, Department of Pathology
²Center for Medical Genetics and Molecular Medicine, Haukeland University Hospital
³Department of Molecular Biology, University of Bergen, Bergen, Norway

Zebrafish (Danio rerio) has been shown to be an attractive vertebrate genetic model for studying hematopoiesis and hematological malignancies. However, methods for detection and isolation of hematopoietic stem cells (HSCs) have not yet been reported. In mammals the combination of Hoechst 33342 staining with flow cytometry can be used for separation of a bone marrow side population (SP), which is highly enriched for HSCs. Considering the similarities of hematopoiesis in teleosts and mammals we applied a similar procedure to hematopoietic kidney marrow cells from adult zebrafish, and identified a segregated cohort of SP cells, that demonstrate a set of features typical of stem cells. SP cells show extremely low scatter characteristics and are small in size with a minimum of cytoplasm. These cells are not labeled by DiD, a specific marker of zebrafish thrombocytes, which strongly suggests that the SP fraction does not include thrombocytes. Treatment of zebrafish kidney marrow cells with reserpine or fumitremorgin C, which inhibit the ABCG2 transporter responsible for Hoechst 33342 efflux, caused a clear reduction in the number of SP cells. Consistent with the quiescent state of HSCs the SP cells are strongly resistant to the myelosuppressive agent 5-fluorouracil. In addition, SP cells specifically demonstrate higher expression of genes known to be markers of HSCs of mammals. Very low expression of genes known as lymphoid and thrombocytic markers in SP cells also confirms that these cells are distinct from zebrafish lymphocytes or mature thrombocytes. Hence, our results have revealed a high evolutionary conservation of the SP phenotype, and the properties of the zebrafish SP cells indicate a significant enrichment for HSCs. Our approach provides the first tool for purification of HSCs from zebrafish that may greatly facilitate genetic analysis of stem cells using the advantages of this vertebrate model.
Nobuyasu Maki

Abstract 25

Nuclear reorganization during dedifferentiation of differentiated cells

Nobuyasu Maki1, Takahiro Murakami1,2, Katsuaki Takechi1,3, Keita Ohsumi4, Yoichi Matsuda5, Kiyokazu Agata1,6

1 Center for Developmental Biology, RIKEN, Kobe, Japan
2 Ehime Women's College, Ehime, Japan
3 Graduate School of Science and Technology, Kumamoto University, Kumamoto, Japan
4 Department of Biological Information, Tokyo Institute of Technology, Yokohama, Japan

It is generally thought that differentiation is an irreversible event, since once differentiated, cells lose their proliferative ability and multipotentiality. However, in some amphibians, differentiated cells revert to stem cell-like cells during regeneration, thereby regaining both proliferative ability and multipotentiality. In the case of newt lens regeneration, pigmented epithelial cells (PECs) in the dorsal iris dedifferentiate to proliferate and transdifferentiate into lens cells. We speculate that nuclear reprogramming must occur in order to gain the proliferative ability and multipotentiality during the dedifferentiation. As the first step for testing this hypothesis, we analyzed the 3D structure of the nucleus and succeeded in detecting the structural changes described below during the dedifferentiation of PECs. These dynamic changes in the nuclear structure suggest that nuclear regulation provides crucial clues for understanding the reversion to stem cell-like cells.

1. Expansion of euchromatic regions
The nuclei of differentiated PECs were small (about 10 µm in diameter) and had a shrunken morphology. The nuclei of differentiated PECs showed highly developed heterochromatic regions stained with anti-tri-methylated histone H3(K9) antibody and small euchromatic regions stained with antibody against acetylated histone H3 and H4. After lentectomy, the nuclei of dedifferentiated PECs became larger (about 20 µm in diameter) and the euchromatic regions expanded.

2. Localization of centromeric heterochromatin regions
In differentiated PECs, centromeric heterochromatin regions, detected by FISH using newt repetitive sequences, had a tendency to be localized in peripheral regions of the nucleus. However, after dedifferentiation, centromeric heterochromatic regions were preferentially localized in central regions of the nucleus.

3. Nucleolar accumulation of nucleostemin
Nucleostemin has been identified in mammals as a nucleolar protein specific to stem cells and cancer cells (Tsai and McKay, 2002). After lentectomy, the expression of nucleostemin was activated and nucleostemin rapidly accumulated in the nucleoli of dedifferentiating PECs 2 days before cell cycle reentry.
René Ketting

Abstract 26

Small RNAs in Germline Development

Saskia Houwing\(^1\), Leonie Kamminga\(^1\), Zhenya Berezikov\(^1\), Angélique Girard\(^2\), Gregory Hannon\(^2\) and René Ketting\(^1\)

\(^1\) Hubrecht Laboratory, Uppsalalaan 8, 3584 CT, Utrecht, The Netherlands
\(^2\) Cold Spring Harbor Laboratories, 1 Bungtown Rd, NY11724, New York, USA

Piwi proteins represent a subclass of the so-called Argonaute proteins: the catalytic engines of small RNA mediated silencing processes. These proteins interact with the small RNAs, using them as guides to identify target mRNA molecules that are subsequently broken down and/or translationally inhibited. The Piwi subfamily displays a very restricted expression pattern. Piwi proteins are expressed in the germline, and are required for fertility. In vertebrates, so far only a role for these proteins has been identified in the testis. Very little is known about the RNA cofactors for piwi proteins, and about their molecular function within the cell.

We here report on the zebrafish Piwi protein Ziwi. Ziwi is present in the cytoplasm of adult germ cells, both male and female. In the testis, Ziwi is expressed in spermatogonial cells, and expression levels decline when meiosis starts. In the ovary, Ziwi is found expressed in oogonia, and in stage I oocytes. In addition, the protein is expressed during embryogenesis in the primordial germ cells (PGCs), where it is localized to perinuclear granules that also contain Vasa protein.

We isolated several fish lines that harbor mutations in the ziwi gene. Animals homozygous for the mutant alleles loose their germ cells between three and four weeks of development, most likely due to excessive apoptosis. In order to find out what is happening in these cells, we are in the process of identifying the RNA cofactors of the ZIWI protein. These turn out to represent a very distinct class of small RNAs, named piRNAs: a very abundant class of approximately 27 nucleotide long RNA molecules, specifically expressed in the germline. These molecules resemble the mammalian piRNAs, but some differences are apparent. A more extensive description of these new, germline specific small RNA molecules will be presented.
Michael Collard

Abstract 27

Deaf-1 Knockout Mice Display Epigenetic Defects in Spermatogenesis, Prostate Cancer, and Alteration of DNA Methylation at Deaf-1 Target Genes

Michael W. Collard, Sara Reardon, and Jodi Huggenvik.

Department of Physiology, Southern Illinois University School of Medicine, Carbondale, IL

DEAF-1 is a developmental transcriptional regulator that maps to human chromosomal region 11p15.5, a region associated with loss of heterozygosity in most solid tumors and epigenetic changes in childhood cancers of the kidney and liver. DEAF-1 binds to DNA containing CpG dinucleotides and may therefore influence DNA methylation at those sites. We have produced Deaf-1 knockout mice and found that mice heterozygous for a Deaf-1 null allele develop prostate lymphoma following a long latency. When mice with a paternal Deaf-1 null allele are bred to wildtype females, they produce offspring that develop defects in spermatogenesis and other organ systems, and these occur in both Deaf-1 heterozygote offspring and their genotypic normal littermates. When females heterozygous for Deaf-1 are bred to wildtype males, testis of male offspring appeared normal indicating the defects in spermatogenesis are corrected when the null allele is passed through the female germline. These data suggest Deaf-1 haploinsufficiency results in parent-of-origin epigenetic defects in germ lines that predispose offspring to infertility and possibly cancer.

To identify target genes that are differentially methylated in Deaf-1 knockout mice, methylated DNA was isolated from normal and homozygous knockout mice at embryonic day 14.5 using recombinant MBD-2 methyl-DNA binding protein, and then labeled with Cy3 or Cy5 dyes. The DNA was hybridized to CpG island microarrays and we identified several gene targets with changes in DNA methylation. One of these target genes, the promoter for the intergenic spacer in the 45S rRNA gene, has been previously identified as a target for altered methylation by Cr(III), and is associated with transgenerational paternal transmission of Cr(III) induced cancers. Bisulfite sequencing of genomic DNA indicates this region is hypermethylated in DEAF-1 knockout mice, suggesting that DEAF-1 may be involved in establishment of methylation patterns at ribosomal genes and possibly other sites in the epigenome. Supported by NIH Grant R01 CA89438A.
Investigation of histone variant localisation in human embryonic stem cells by transfection of GFP fusion protein mRNA

Margaret L. Shaw¹, Evan J. Williams², Jeff Craig¹, K.H. Andy Choo², Richard Saffery¹

¹ Epigenetics Research, Murdoch Childrens Research Institute, Royal Childrens Hospital, Parkville VIC 3052 Australia
² Chromosome and Chromatin Research, Murdoch Childrens Research Institute, Royal Childrens Hospital, Parkville VIC 3052 Australia

Human embryonic stem cells (hESCs) have the ability to differentiate into cells from all three germ layers. The differentiation process involves the coordinated regulation of gene expression most likely mediated by epigenetic remodelling of hESC chromatin. We are interested in studying the epigenetics of early stem cell differentiation, and in particular investigating the changes in distribution of histone variants within the nucleus pre and post differentiation induction. We have produced a panel of histone variant-GFP fusion expression DNA constructs for histone variants H2AX, H2AZ, H2Abbd, macroH2A, TSH2B, and H3.3, as well as histone H1 variants, and control bulk histones. These have been used to produce in vitro transcribed messenger RNAs that we have transfected into hESCs to investigate histone variant-GFP localisation in undifferentiated cells or cells exposed to retinoic acid for 24 hours. As a positive control, cells we transfected cenpA-GFP mRNA, and demonstrated specific centromeric signals. Using this approach we have observed the novel localisation of a number of histone variants, including H2Abbd and bulk histone H3 in hESCs. Furthermore, transfection of macroH2A-GFP revealed the presence of an inactive X chromosome in this cell line.

In conclusion, we have demonstrated the feasibility of fusion protein mRNA transfection as a means of investigating sub-nuclear localisation of chromatin proteins. This is likely to be a very useful technique for investigating the localisation of novel proteins in hESCs that are refractory to DNA transfection.
Johan Jakobsson

Abstract 29

Lentiviral mediated shRNA knock-down of the transcriptional co-repressor KAP-1 impairs neural differentiation of murine embryonic stem cells

Johan Jakobsson, Maciej Wiznerowicz, Didier Trono

Laboratory of Virology and Genetics, School of Life Science, EPFL, Lausanne, Switzerland

KRAB-zinc finger proteins are a large family of transcriptional repressors with approximately 300 members present in the human genome. Members of the protein family are characterised by a DNA binding zinc finger domain and a KRAB domain. The KRAB domain is essential for repression and is absolute dependent on the co-repressor KAP-1 (also known as Tif1b). Mice lacking Kap1 die during early post implantation development (E5.5) suggesting an important role for KRAB-zfp during embryogenesis. In order to study the role of KAP-1 during early development we have transduced murine ES-cells with a lentiviral vector expressing shRNA directed towards KAP-1. Transduction with such lentiviral vectors lead to a reduction of cellular KAP-1 protein with more than 70%. The KAP-1 knock-down cells appear to have a normal phenotype and are not different to control cells in regards to proliferation. In order to investigate the role of KAP-1 during ES cell differentiation we decided to use a well established monolayer culture protocol (Ying et al, Nat Biotech 2003). This protocol leads to homogenous differentiation towards neural progenitor cells allowing for analysis of a single lineage. In order to monitor differentiation we have used transgenic ES-cell lines expressing GFP under the Oct4 and Sox1 promoter. We found that the KAP-1 cells are deficient in differentiation at a step that appears to be after Oct4 expression is downregulated. We are currently investigating the mechanism behind this phenotype.
Amy Hazen

Abstract 30

A role for SHIP in homing and retention of HSC in the BM

Amy L. Hazen, Caroline Desponts, and William G. Kerr

Cancer Biology Program, Moffitt Cancer Center and Research Institute

SH2-containing-5’-inositol-phosphatase (SHIP) can influence proliferation, survival or differentiation in hematopoietic cells. Targeted disruption of SHIP results in an expansion of NK cells and certain myeloid subsets demonstrating that SHIP is a crucial mediator of signaling in the hematopoietic compartment. Previously we showed that SHIP-deficiency also permits an expansion of hematopoietic stem cells (HSC) in germline SHIP-/- mice. We observed that SHIP-/- mice contained significantly more HSC in their bone marrow, spleen and peripheral blood. However, SHIP-/- HSC failed to effectively compete with WT HSC for long-term multi-lineage repopulation of the hematopoietic compartment. We found that germline SHIP-/- HSC do not home or are not retained as efficiently in bone marrow (BM) as compared to WT HSC (Desponts et al Blood 2006). These findings indicate compromised repopulation capacity may be due to changes in surface receptors that determine chemotaxis and adhesion to BM stroma. Consistent with this we found that CXCR4 and VCAM1 are both down-regulated on SHIP-/- HSC. Here we show that in situ deletion of SHIP in HSC in BM chimeras does not harm long-term multi-lineage repopulation. These in vivo deleted recipients show short and long-term hematopoietic repopulation by SHIP-/- HSC demonstrating that SHIP-deficiency does not harm the long-term multi-lineage repopulation for resident HSC. These findings provide further support that SHIP is required for proper BM homing of HSC following intravenous infusion – the primary means for experimental and clinical BMT procedures. Further understanding the role that SHIP plays in control of chemotaxis and homing receptor expression should provide useful insights into the molecular processes affecting homing and retention of HSC to the BM niche that supports long-term repopulation and self-renewal.
Alysson Muotri

Abstract 31

L1-mediated somatic mosaicism in neuronal precursor cells

Alysson R. Muotri & Fred H. Gage

Laboratory of Genetics, The Salk Institute, 10010 N. Torrey Pines Rd, La Jolla, CA 92037
E-mail: muotri@salk.edu

Understanding what produces neuronal diversification has been a longstanding challenge for neuroscientists. The recent finding that LINE-1 (Long Interspersed Nucleotide Elements-1 or L1) retroelements are active in somatic neuronal progenitor cells provided an additional mechanism for neuronal diversification (Muotri et al Nature, 2005 and Muotri & Gage, Nature 2006). Together with their mutated relatives, retroelements sequences constitute 45% of the mammalian genome with L1 elements alone representing 20%. The fact that L1 can retrotranspose in a defined window of neuronal differentiation, changing the genetic information in single neurons in an arbitrary fashion, allows the brain to develop in distinctly different ways. This characteristic of variety and flexibility may contribute to the uniqueness of an individual brain. However, the extent of the impact of L1 on the neuronal genome is unknown. The characterization of somatic neuronal diversification will not only be relevant for the understanding of brain complexity and neuronal organization in mammals but may also shed light on the differences in cognitive abilities, personality traits and many psychiatric conditions observed in humans.
Epigenetic gene regulation by DNA methylation and histone tail modifications plays a central role in normal mammalian development. During development, epigenetic marks are substantially reprogrammed in the zygote, preimplantation embryo, and in primordial germ cells (PGCs). Reprogramming plays important roles in imprinting and in the acquisition of the pluripotential state of embryonic and stem cells. Reprogramming includes the erasure of DNA methylation in imprinted and non-imprinted gene sequences. We are investigating a candidate mechanism for erasure of DNA methylation, and can show that targeting of a candidate enzyme to a methylated region in vivo in a transgenic system can lead to its efficient demethylation. We are also carrying out genome wide screens of DNA methylation in pluripotent tissues as compared to differentiated tissues, in order to gain insights into the epigenetic regulation of the pluripotent state. Some pluripotency genes appear to be methylated in mature germ cells and this methylation is erased in pluripotent cells. Collectively, our observations suggest possible new approaches to regenerative medicine and cancer therapy.
Carlos Pereira

Abstract 33

Chromatin events underlying lymphocyte reprogramming to pluripotency

Carlos F. Pereira¹, Remi Terranova², Natalie Ryan¹, Wei Cui³, Matthias Merkenschlager¹, Amanda G. Fisher¹

¹ Lymphocyte Development Group, MRC Clinical Sciences Centre, Imperial College School of Medicine, Hammersmith Hospital, Du Cane Road, London W12 0NN, UK.
² Present address: Friedrich Miescher Institute for Biomedical Research, Maulbeerstrasse 66, 4058 Basel, Switzerland.
³ Stem Cell Initiative, Institute of Reproductive and Developmental Biology, Faculty of Medicine, Imperial College London, Hammersmith Hospital, Du Cane Road, London W12 0NN, UK.

Although differentiated cells normally retain cell-type-specific gene expression patterns throughout their lifetime, cell identity can sometimes be modified or reversed in vivo by transdifferentiation, or experimentally through cell fusion or by nuclear transfer. To define the epigenetic changes required for somatic cell reprogramming to a pluripotent state, we generated heterokaryons between human B-lymphocytes and mouse embryonic stem (ES) cells. We show that within two days of heterokaryon formation, lymphocyte nuclei adopt a structure resembling ES cells and initiate the expression of several pluripotency-associated genes such as hOct4, hNanog, hCripto, and hRex1. This de novo expression is coordinated with the shut down of several lymphocyte-associated genes, but neither apparently requires DNA replication or nuclear fusion. The level of ES-associated gene expression in reprogrammed lymphocytes gives a profile that is strikingly similar to human ES cells and distinct from the expression profile of mouse ES cells. Moreover, genes encoding FGF signalling pathway components (hFgfr1 and hFgf2), required to maintain pluripotency in human ES cells but not in mouse ES cells, were strongly upregulated upon cell fusion. However, four out of 13 ES-associated genes analysed (hSox2, hTert, hFoxd3 and hDppa4) were not easily re-activated in heterokaryons and three of these display heterochromatic features such as late replication and repressive histone marks. This closed chromatin configuration may offer an explanation as to why these genes are difficult to re-activate. Collectively our results underscore the importance of the chromatin environment modulating transcriptional changes in somatic cells induced by cell fusion with ES cells. Finally, it provides a novel system for investigating the underlying mechanisms of genome reprogramming and acquisition of pluripotency.
Laura O'Neill

Abstract 34

Comparison of histone modifications on key regulatory genes during early mouse development using C-ChIP

L.P.O'Neill, Matthew VerMilyea and Bryan Turner

Chromatin and Gene Expression Group, IBR building, Medical School, Birmingham University B15 2TT

The epigenetic code is becoming more complex, with diverse modifications to the N-terminal tails of the core histones correlating with specific aspects of gene regulation. The modification itself, the histone involved and the amino acid residue are all important determinants of these epigenetic marks. We have used both mouse and human models to investigate the epigenetic marks present during early embryonic development. Embryonic stem cell lines (ES cells) which are derived from the inner cell mass (ICM) of the preimplantation blastocyst are pluripotent and maintained in this state by a few key regulator genes, whose expression is controlled by epigenetic mechanisms. Enzyme-catalyzed modification of the core histones packaging DNA, is a central component of these mechanisms, and can be assayed on selected genes by chromatin immunoprecipitation (ChIP). Since conventional ChIP requires about 10 million cells, it cannot be applied to the early embryo (100 cells or less) and it has not been possible to establish whether or not ES cells provide a good epigenetic model of the ICM. Here we describe a novel ChIP procedure (C-ChIP) based on the use of Drosophila carrier chromatin, that allows us to define and quantify patterns of histone modification in fewer than 100 cells. We show that well-established activating and silencing marks (H4 acetylation, H3K4 tri-methylation and H3K9 di-methylation) are similarly distributed across regulator genes such as Nanog and Oct4 in ICM and ES cells, while other modifications provide evidence for epigenetic differences.
Embryonic stem (ES) cells have two qualities that make them developmentally and clinically important: the ability to self-renew and the ability to differentiate into any embryonic cell type (pluripotency). A lot of effort has gone into defining molecular requirements of self-renewal, but little is known about how cells commit to differentiation. Even less is known about the rare molecular events that occur when pluripotent cells are cultured in vitro to give rise to ES cells. The difference between pluripotent cells and the lineage-committed cells derived from them, or their immortalised derivatives in vitro (i.e. ES cells) must be, by definition, epigenetic. Consistent with this assertion, several epigenetic silencing factors have been shown to be important for early embryonic viability in mice, although it is not always clear why the mutant embryos do not survive. I will describe our current work on examining the function of the Nucleosome Remodelling and Histone Deacylation (NuRD) co-repressor complex in pluripotent cells, and show evidence that NuRD-mediated epigenetic silencing is required for development and lineage commitment of pluripotent cells in vivo and ex vivo.
Dnmt3a-G9a complex represses differentiation genes to maintain self-renewal of neural stem cells

Hao Wu, Volkan Coskun, En Li, Guoping Fan, Yi Eve Sun

Departments of Molecular & Medical Pharmacology and Psychiatry & Behavioral Sciences, MRRC at UCLA Neuropsychiatric Institute
Department of Human Genetics, UCLA School of Medicine, NRB room 351, 635 Charles E. Young Drive South, Los Angeles, CA 90095

Mechanisms by which stem cells self-renew while maintaining their differentiation potential are linked to heritable epigenetic modifications of differentiation genes. The repressed yet poised chromatin structure of key developmental genes in embryonic stem cells (ESCs) is partly maintained by polycomb group (PcG) complexes, and is required for the maintenance of pluripotency. However, PcG-mediated repression is released during ESC differentiation, suggesting that additional epigenetic machineries mediate repression of poised differentiation genes in self-renewable tissue-specific stem cells. Here we report that during the transition from ESCs to multipotent neural stem/progenitor cells (NPCs), repressive epigenetic marks in poised glial differentiation genes switch from PcG-mediated tri-methylation of lysine 27 on histone H3 (H3K27me3) to di-methylation of lysine 9 on histone H3 (H3K9me2); this switch is associated with increased DNA cytosine methylation by the de novo DNA methyltransferase Dnmt3a. Surprisingly, Dnmt3a reversibly represses genes by DNA methylation and by a novel, DNA methylation independent mechanism that involves recruitment of the histone H3-K9 methyltransferase G9a. Deficiency in Dnmt3a or G9a disables the self-renewal capacity of NPCs and causes their spontaneous differentiation into glial cells; in Dnmt3a-null NPCs, this phenotype is partially rescued with an enzymatically inactive Dnmt3a mutant that still interacts with G9a. Our data thus indicate that the Dnmt3a-G9a complex establishes repressive marks on poised glial lineage genes to maintain NPCs in an undifferentiated state, suggesting that reversible repression of poised differentiation genes is a common epigenetic strategy for stem cell maintenance, but mediated by distinct epigenetic machineries in ESCs and somatic stem cells.
Jurgen Knoblich

Abstract 37

*Drosophila* as a model for stem cell derived tumor formation

Joerg Betschinger, Ralph Neumueller and Juergen A. Knoblich

IMBA, Dr Bohr Gasse 3, 1030 Vienna, Austria

Stem cells can generate self-renewing and differentiating daughter cells at the same time. We are using *Drosophila* as a model system to understand how they control the balance between these two fundamentally different types of progeny. Using a proteomics approach for proteins that segregate into one of the two daughter cells in neuroblasts (stem cell like precursors of the central nervous system) we have found the growth regulator *brat* (*brain tumor*). During mitosis, Brat segregates into one of the two daughter cells, where it downregulates protein synthesis, stops proliferation and prevents cell growth. In *brat* mutant animals, all daughter cells of the stem cell undergo self renewal and continue to proliferate. This leads to dramatic overproliferation and the formation of a stem cell derived tumor which grows indefinitely and kills the animal. Tumors will continue to proliferate indefinitely, even when transplanted into other flies, thus indicating that cells become immortalized. Very similar phenotypes are observed in flies mutant for Lethal (2) giant larvae (Lgl), where Brat is present but does not segregate asymmetrically. Thus, the asymmetric segregation of Brat into one of the two daughter cells regulates proliferation in *Drosophila* neural stem cells.

Brat is a member of a conserved protein family characterized by a similar domain composition. We have analyzed the function of other family members and find that they regulate self renewal in other types of stem cells. Like in *brat* mutants, stem cells overproliferate and form of tissue specific tumors. Our results indicate that this role might also be conserved in vertebrates suggesting that *brat*-like proteins have a conserved role in regulating stem cell self renewal.
A single type of progenitor cell maintains normal epidermis

Elisabeth Clayton¹, David P Doupé¹, Allon M. Klein², Douglas J Winton³, Benjamin D Simons², Philip H Jones¹

¹ MRC Cancer Cell Unit, Hutchison-MRC Research Centre, Cambridge CB2 2XZ, UK
² Cavendish Laboratory, University of Cambridge, Madingley Road Cambridge CB3 0HE, UK.
³ Cancer Research UK Department of Oncology, University of Cambridge, Cambridge Institute

According to the current model, adult epidermis is maintained by two discrete populations of progenitor cells. These comprise self-renewing stem cells and their progeny, known as transit amplifying (TA) cells, which go on to differentiate after several rounds of cell division. We have used inducible genetic labelling to track the fate of a representative sample of progenitor cells at single cell resolution for a year in vivo. By analysis of clone size distributions we show that normal epidermis is maintained by only one type of progenitor cell which undergoes asymmetric division at a rate that ensures epidermal homeostasis. We challenged the predictive power of this new paradigm in epidermis rendered highly proliferative by retinoic acid treatment, and found that the model successfully predicted the observed clone size distribution. These results raise important questions on the role of stem cells in vivo and on the development of cancer.
Phillip Karpowicz

Abstract 39

DNA is Partitioned Asymmetrically in the Germ Stem Cells of the Drosophila ovary

Phillip Karpowicz¹, Milena Pellikka², Dorothea Godt², Ulrich Tepass², Derek van der Kooy¹

¹ Department of Medical Genetics and Microbiology
² Department of Zoology, University of Toronto, Toronto, Canada

The Immortal Strand Hypothesis proposes that asymmetrically dividing stem cells segregate chromosomes non-randomly to retain ancestral DNA templates. This asymmetric segregation of DNA was investigated in germline stem cells of the Drosophila ovary, which divide only asymmetrically generating a new stem cell and a differentiating cystoblast. Asymmetric partitioning of DNA occurs in germline stem cells in vivo. When germ stem cells are forced to differentiate by overexpression of Bag of Marbles, no asymmetric partitioning of DNA is observed between germ cell progenitors, showing that this process only occurs in stem cells and not their progeny. Moreover, when asymmetric division is disrupted the uneven partitioning of DNA does not occur. Ectopic expression of Decapentaplegic, a BMP family morphogen that causes both daughter cells to adopt stem cell identity, abolishes this asymmetric partitioning of DNA. Similar disruption is observed in Bag of Marbles mutants whose inability to differentiate into cystoblasts also abolishes the asymmetric partitioning of DNA. These results support chromosome cosegregation in germ stem cells in vivo and show that cosegregation is coupled to mechanisms specifying cellular differentiation via asymmetric cell division.
The possibility to extract and induce proliferation of human stem cells obtained from a plain adult skin biopsy, and to induce cell differentiation into neurons, opens up considerable perspectives within the field of neurosciences, including novel approaches for the treatment of neurodegenerative diseases. It is now becoming feasible to amplify an autologous neuronal stem cells population isolated from a patient’s small skin biopsy, and to implant it back to the patient soon thereafter. In the literature, however, it is not yet clear if those neuronal precursors can differentiate into fully mature neurons. Our objective is to demonstrate that these cells can differentiate into neurons expressing late differentiation markers. Skin samples obtained from breast reductions are incubated overnight in 250µg/ml of thermolysin at 4 degrees Celcius. The epidermis layer is peeled off the dermis layer and the samples are submitted to a 1h trypsin treatment at 37 degrees Celcius. The cells hereby extracted are plated for 28 days in a DMEM/F-12 (3:1)-based proliferation medium, and then for 21 days in a DMEM/F-12 (3:1)-based differentiation medium. The cells are fixed in formol/methanol, after which immunohistochemistry and flow cytometry analyses are performed. Stem cells extracted from adult human skin form clusters-like neurospheres that hold a proliferative capacity demonstrated by BrdU marking. After 3 passages, cells within the neurospheres are 70% nestin-positive and 20% neurofilament M-positive. Following cluster fragmentation and cells spreading, the latter are expressing MAP-II, neurofilament M, β-III-Tubulin and synaptophysin after 1 week of differentiation. We have demonstrated that adult human skin stem cells can differentiate into mature neurons, expressing specific late differentiation markers such as PSD-95, NeuN and neurofilament H. Various cellular and non-cellular coatings are currently under investigation in order to determine and stimulate optimal differentiation of our adult human stem cells into neurons, as electrophysiology studies are presently underway to evaluate the in vitro functionality of those neurons.
Stuart Avery

Abstract P2

An inducible RNAi system in human embryonic stem cells to knockdown SMAD proteins.

Stuart Avery, Gaetano Zafarana and Peter Andrews

Centre for Stem Cell Biology, University of Sheffield, Western Bank, Sheffield, S10 2TN. UK

The proliferative and pluripotent capacity of human embryonic stem cells (hESCs) provides for their use as a powerful tool in the development of therapeutic applications for a range of degenerative diseases. Understanding the external factors and signalling pathways required to maintain self-renewal in hESCs is crucial to the development of efficient serum-free and ‘feeder’-free culture conditions, devoid of animal products.

TGF-β family signalling can be subdivided into TGF-β/Activin/Nodal signalling and the BMP/GDF pathways. TGF-β/Activin/Nodal signalling is associated with the activation of the SMAD-2/3 pathway which has been deemed necessary in maintaining pluripotency in hESCs. Conversely, BMP signalling activates SMAD-1/5/8 and has been shown to differentiate hESCs towards extra-embryonic lineage. Both SMAD-2/3 and SMAD-1/5/8 require dimerisation to the co-SMAD SMAD-4 for nuclear translocation.

A number of serum-free and feeder-free systems have been developed which either use activin/nodal in combination with bFGF, or noggin (a ligand trap of BMP) and a higher dose of bFGF. The mechanism that is supporting self-renewal is not clearly understood. We have developed an inducible RNAi system that can be used to knockdown target genes in hESCs. This system has been used to successfully knockdown a number of key SMAD genes in a controlled fashion. We are currently addressing the role of individual SMAD components, to dissect the downstream targets of TGF-β signalling that are involved in controlling self-renewal.
Manjiri Bakre

Abstract P3

Wnt directs differentiation of embryonic stem cells to mesendoderm

Manjiri M. Bakre*, A. Hoi, Li Pin, Yvonne Koh, Z. Kelly and L Stanton

60, Biopolis St., Genome # 02-01, Genome Institute of Singapore, Singapore: 138672

Embryonic stem (ES) cells have the unique capability to give rise in vitro to cell types of all three germ lineages–ectoderm, mesoderm, and endoderm. Through comprehensive gene expression analysis of mouse and human ES cells we have found that ES cells express the components of the Wnt signaling pathway. Wnt ligands act as morphogens in specification of mesoderm and endoderm in embryo, but paradoxically have been implicated in maintaining pluripotency of embryonic stem (ES) cells.

We have investigated the role of Wnt signaling on mouse and human ES cells in vitro. ES cells were treated with Wnt3A or an inhibitor of GSK3-beta to activate the canonical Wnt pathway. We have found that Wnt pathway activation induced differentiation of ES towards the meso/endodermal lineage. Differentiation status was established by examining the expression of numerous cells markers that delineate lineages by RT-PCR, immunostaining, and microarray analysis. The differentiated ES cells continue to express Oct4 and Nanog, markers of pluripotency. We did not detect any differentiation along the neuroectoderm lineage. Thus, we have found that long term passage of ES cells under sustained Wnt activation has yielded mouse and human progenitor cell lines that are multipotential, but restricted to meso/endodermal differentiation. We have isolated single cell clones to further differentiate these multipotential cells to organ specific cells and such attempts are underway in our laboratory.
Leonora Buzanska

Abstract P4

Neural differentiation and electrophysiological properties of human umbilical cord blood neural stem cell line (HUCB-NSC)

Buzanska L., Jurga M., Sobanski T., Coecke S., Domańska-Janik K.

1NeuroRepair Department, Medical Research Institute, Warsaw, Poland, 2ECVAM, 3BMS Joint Research Center, Ispra, Italy

First, non-immortalized the expanding population of neural stem/progenitor cells was selected from non-hematopoietic (CD34 and CD45 negative) mononuclear fraction of human cord blood – HUCB-NSC (Buzanska et al. Stem Cells & Dev 2006). These cells can be differentiated into neuronal, astrocytic and oligodendroglial-like cells. Upon treatment with dBcAMP more than 80% of nestin-expressing HUCB-NSC differentiated into neuron–like cells. This was accompanied by expression of several functional proteins including GluR2, dopamine, serotonin and acetylcholine receptors, Gad 65, GABARAPL1 and 3; SV2 and TH as revealed by gene microarray and immunocytochemical. To address the issue of functional properties of HUCB-NSC we used whole cell patch clamp recordings. Two types of voltage-sensitive currents were found in differentiated HUCB-NSC: (1) inward rectifying potassium current (Kir) and (2) outward rectifying potassium current (IK+). Kir could be reversibly blocked by external Cs+ and Ba2+. The reversal potential of Kir was shifted when the external potassium concentration changed. IK+ could only be recorded from approximately 50% of differentiated cells, but not from undifferentiated HUCB-NSC. IK+ could be reversibly blocked by TEA and 4-AP, but not by externally applied Cs+. Differentiated HUCB-NSC also expressed glutamate, serotonin and dopamine receptors. Kainic acid, serotonin and dopamine showed apparently suppressing effects on Kir channels in most of the differentiated HUCB-NSC. This effect was blocked by the specific receptor antagonists. The action-potential was recorded from HUCB-NSC cells when differentiated directly on the multielectrode array chips. The role of bHLH transcription factors during differentiation of HUCB-NSC was also introduced. Over-expression of Id1 resulted in de-differentiation of the HUCB-NSC cells. In contrast, transfection with antisense Id1 resulted in promotion of neuronal differentiation. Thus the influence of Id1 protein on neural differentiation of HUCB-NSC is unique and selectively referred to neurogenesis inhibition. These results suggest that HUCB-NSC could conceivably be differentiated into functional neuron-like cells.
Marco Cassano

Abstract P5

Magic F1, a dimerized c-met binding domain, induces muscle hypertrophy by protecting from apoptosis myogenic progenitors.

Cassano Marco¹, Stefania Crippa¹, Biressi Stefano¹, Michieli Paolo¹, Allegretti Marcello¹, Cusella De Angelis Gabriella², Comoglio Paolo¹, Cossu Giulio¹ and Sampaolesi Maurilio¹, ²

¹ Stem Cell Research Institute, H. S. Raffaele, Milan; ²Human Anatomy Institute, Dept. of Exp. Med., University of Pavia, Italy.

Hepatocyte growth factor (HGF), also referred to as scatter factor (SF), is an important paracrine mediator of epithelial-mesenchymal cell interactions (Birchmeier C & Gherardi E). It is secreted by mesenchymal cells and affects cell proliferation, motility and morphology. These diverse biological activities are a result of HGF/SF binding to and activating its high affinity tyrosine kinase receptor called c-Met. HGF/SF was the first growth factor shown to be able to stimulate activation and early division of adult satellite cells in culture and in muscle tissue. MAGIC F1 (cMet activating genetically improved chimeric factor 1) is a human recombinant protein HGF-related, containing the leader sequence for protein secretion, a hairpin loop sequence and a combination of double repeated NK1 and NK2 functional kringle domains, where is located the high-affinity binding site for c-Met. It is able to activate c-Met receptor, stimulate AKT pathway and induce myocyte hypertrophy both in vitro and in vivo. Moreover, the AKT phosphorylation preserves myocytes from apoptotic events during muscle regeneration as revealed in a transgenic animal model expressing MAGIC F1 under the dependence of a skeletal muscle-specific promoter. These results showed clearly a starting point for clinical implication of MAGIC F1 recombinant protein, since inducing muscle hypertrophy could be a strategy to oppose the degenerative process of muscular pathologies.

Hepatocyte growth factor (HGF), also referred to as scatter factor (SF), is an important paracrine mediator of epithelial-mesenchymal cell interactions. It is secreted by mesenchymal cells and affects cell proliferation, motility and morphology. These diverse biological activities are a result of HGF/SF binding to and activating its high affinity tyrosine kinase receptor called c-Met. HGF/SF was the first growth factor shown to be able to stimulate activation and early division of adult satellite cells in culture and in muscle tissue. MAGIC F1 (cMet activating genetically improved chimeric factor 1) is a human recombinant protein HGF-related, containing the leader sequence for protein secretion, an hairpin loop sequence and a combination of double repeated NK1 and NK2 functional kringle domains, where is located the high-affinity binding site for c-Met. It is able to activate c-Met receptor, stimulate AKT pathway and induce myocyte hypertrophy both in vitro and in vivo. These results showed clearly a starting point for clinical implication of this recombinant protein since inducing muscle hypertrophy is a novel strategy to oppose the degenerative process of muscular dystrophy.
Human neural precursor cells can be derived currently by a number of methods, however none are clonal and most involve many steps and considerable time. We have developed a simple, clonal method of generating neural stem cells and progenitors directly from human embryonic stem cells. Human embryonic stem cells exhibit pluripotentiality in their ability to form derivatives of all three embryonic germ layers. We have determined that in the absence of external signals, human embryonic stem cells default to a neural identity. When single human embryonic stem cells were placed in serum-free media, they rapidly acquired a neural precursor identity as observed by the downregulation of the pluripotency marker Oct-4 and upregulation of the neural precursor markers sox-1 and nestin. Using these minimal serum-free conditions, we plated single H9 and CA1 human embryonic stem cells at clonal densities, and floating sphere colonies termed 'neurospheres' arose from 0.14% of the starting population. These neurospheres contained a mixture of neural stem cells and their progenitors. RT-PCR analysis of neurospheres showed expression of the neural precursor markers nestin and sox-2, but not the early endodermal and mesodermal markers HNF3-beta or brachyury. Neurosphere colonies, generated from a single primitive neural stem cell, were differentiated to form beta-3-tubulin positive neurons and O4-positive oligodendrocytes by immunocytochemical staining, illustrating multipotentiality, and passaged to form clonal secondary spheres, exhibiting self-renewal.
Lingyi Chen

Abstract P8

Nanog and Cdx2 antagonize each other in the segregation of the inner cell mass and the trophectoderm

Lingyi Chen, Chi-Wei Lu, and George Q. Daley

Childrens Hospital Boston and Harvard Medical School, 300 Longwood Ave, Karp 7004, Boston, MA 02115

The formation of a multi-cellular organism from a single cell, the zygote, requires multiple steps of cell differentiation. The very first cell fate decision in the early pre-implantation embryo is the segregation of trophectoderm from the inner cell mass (ICM) during the transition from morula to blastocyst. This segregation is marked by the reciprocal expression pattern of Cdx2 in the trophectoderm, and Nanog and Oct4 in the ICM. Recent studies have shown that Oct4 and Cdx2 form a repressive complex that reciprocally regulates their gene expression, while Nanog is believed to act at a later stage to specify the fate of the epiblast.

We studied trophectoderm differentiation in an in vitro system, in which mouse embryonic stem (ES) cells are induced to differentiate into trophoblastic stem (TS) cells by ectopic expression of an activated H-Ras allele. First, we found that Nanog and Cdx2 are reciprocally regulated upon H-Ras induction, while Oct4 expression remains unchanged. Second, up-regulation of trophectodermal markers can be quantitatively prevented by Nanog over-expression. Third, the knock-down of Nanog not only leads to up-regulation of an extraembryonic endoderm marker, Gata6, but also enhances the expression of Cdx2. Last, chromatin immunoprecipitation experiments and luciferase reporter assays demonstrated that Nanog and Cdx2 bind to and repress each other’s promoter. In summary, our data suggest that Cdx2 may specify trophectoderm fate by antagonizing Nanog as well as Oct4.
Human embryonic stem cells (hESCs) are pluripotent cells derived from embryos at the blastocyst stage. Their embryonic origin confers upon them the ability to proliferate indefinitely in vitro while maintaining the capacity to differentiate into a large variety of cell types. Our group has recently shown that the addition of Activin or Nodal in a chemically defined medium is sufficient to maintain pluripotency of hESCs. On the other hand, inhibition of Activin/Nodal signaling drives differentiation of hESCs towards neuroectoderm (Vallier et al., 2004, and our unpublished data). Together, these results suggest that Activin/Nodal signaling pathway maintains the pluripotent status of hESCs by blocking neuroectoderm differentiation. To further understand the molecular mechanisms involving Activin/Nodal signaling in pluripotency, we are studying in hESCs the function of key Smad-binding partners which are known to play a role in neuroectoderm specification. One of these is TIF1γ, a member of the Transcriptional Intermediary Factor 1 (TIF1) family which inhibits mesendoderm differentiation during Xenopus development. TIF1γ is reported to promote neuroectoderm differentiation by enhancing proteosomal degradation of Smad4, a common Smad mediator of the TGFβ/Activin/Nodal and BMP4 signaling cascade (Dupont et al., 2005). The function of TIF1γ appears to be conserved in mammalian cells since its overexpression is capable of blocking the anti-proliferative effect of TGFβ on cancerous cell lines. Interestingly, TIF1γ has also a function in cell fate commitment of human hematopoietic stem cells which is to mediate erythrocyte differentiation by binding phosphorylated Smad2/3 (He et al., 2006).

Our preliminary studies show that TIF1γ is highly expressed in hESCs, with the protein localized predominantly in the nucleus, and that TIF1γ binds Smad4 as well as phosphorylated Smad2/3. By overexpressing TIF1γ in hESCs, we found that it does not block TGFβ/Activin/Nodal or BMP4 signaling. Indeed, luciferase reporter assays show that raising the levels of TIF1γ in hESCs increases the transcriptional response to Activin signaling without affecting pluripotency. Finally, differentiation of TIF1γ-overexpressing hESCs shows that TIF1γ is a key mediator of TGFβ gene responses during cell fate determination. These findings underscore the importance of Activin/Nodal signaling in hESCs and provide additional insights into its mechanisms of action in pluripotency and differentiation.

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Abstract P10

MEF characterization to identify growth factors important for ESC self-renewal.

Cleo Choong¹, Boon Seng Soh², Henry Yang³, Bing Lim² and Mahendra Rao¹

¹ Laboratory of Stem Cell Biology, Singapore Stem Cell Consortium, Singapore 138667; ² Stem Cell and Developmental Biology Group, Genome Institute of Singapore, Singapore 138672; ³ Stem Cell Biology Group, Bioinformatics Institute, Singapore 138671

Examination of potential self-renewal maintenance ability of fibroblasts that support embryonic stem cells (ESCs) has suggested that at least some of this capability is due to the presence of soluble components that might either inhibit cell death, enhance proliferation or inhibit differentiation. While mouse embryonic fibroblasts (MEFs) appear to be capable of supporting most ESC populations this property appears to be specific to a specific stage of fibroblast development and appears to be enriched in some strains of mice. In addition, examination of proliferating hESC cultures has suggested that several aspects of the culture are important in maintaining ESCs grown in MEF conditioned media. These include components present in the substrate on which the ESCs grow on, factors made by the ESCs themselves as suggested by density and clonal efficiency studies, secreted factors provided by the MEFs as suggested by conditioned media experiments and the receptors that are have been found on the hESCs. In this study, we describe a parallel and complementary bioinformatics approach to identify candidate signaling pathways that may be active when ESC are propagated on MEFs. We have developed datasets of whole genome profiling of MEFs and of multiple lines of mouse and human ESC using Illumina bead arrays and MPSS. We have identified growth factors secreted at detectable levels and correlated those with receptors present on ESC and developed a list of receptor-ligand pairs that may signal self-renewal. Various perturbation experiments were then carried out to show that the activation of a combination of identified growth factors is sufficient to maintain ESC self-renewal.
Stem cells possess the characteristic of long-term self-renewal which allows them to proliferate for an extensive period of time without differentiation. The cell cycle structure of mouse embryonic stem cells (ESCs) was observed to be different from mouse fibroblast with prolonged S phase and truncated gap phases. ESC differentiation was reported to be related to the activation of Rb-E2F pathway. These suggest the importance of cell cycle pathways and their associated molecules in maintaining self-renewal properties of ESCs. To better understand the activation of cell cycle pathways relevant for self-renewal, we integrate several gene expression data sets of ESCs and embryoid bodies (EBs). Using those integrated data sets, we perform gene set enrichment analysis. In this analysis, we dissect cell cycle pathways into smaller paths and identify the activated/deactivated paths in ESCs and EBs. To increase the confidence of our result, we hyphenate the gene set enrichment analysis with clustering techniques. We have identified several activated/deactivated paths in cell cycle pathways. Some paths agree with the published literatures while some novel paths are discovered and need to be experimentally verified. The results indicate that specific parts of the cell cycle control mechanism may be important for the continuation of ESCs self-renewal.
Control of differentiation/proliferation in cortical neural progenitor stem cells by specific combinations of transcription factors and co-factors

Peter Cook, Derek Solum, M. Geoff Rosenfeld

Room 345, CMM West, School of Medicine, 9500 Gilman Drive, Mail Code 0648
University of California, San Diego, La Jolla, California 92037-0648

The pyramidal neurons of the developing cortex are divided into discrete layers characterized by established layer marker genes. The development of these different neuronal subtypes is controlled by cell-autonomous factors functioning in the undifferentiated neural progenitor cells of the ventricular zone. One such factor is the forkhead DNA-binding transcription factor FoxG1, which is involved in repression of Cajal-Retzius cell fate in cortical neurons not destined for the marginal zone. We chose to address the molecular mechanism by which FoxG1 functions and to identify relevant target genes using primary monolayer cell culture systems. We have seen that FoxG1 is expressed in the nucleus of all cortical progenitor ("neural stem") cells and in the vast majority of post-mitotic cortical neurons. Furthermore, FoxG1 expression in post-mitotic neurons is mutually exclusive with markers for Cajal-Retzius cells. FoxG1 was seen to occupy a forkhead binding site on the p21 promoter in neural progenitor cells. In addition, we have identified a novel interaction between FoxG1 and the lysine-specific demethylase enzyme LSD1, a component of multiple co-repressor complexes, suggesting a possible molecular mechanism for FoxG1-mediated repression of target genes.
In ES cells, lower levels of Oct4 facilitate entry into the neural lineage

During gastrulation, the epiblast sorts into germ layers prior to lineage specification. Our interest is in knowing what molecular profile facilitates entry into the neural lineage. Given that Oct4 is essential for development of the epiblast, maintenance of pluripotent undifferentiated ES cells, a mediator of cell fate, and has family members with established roles in neural fate decisions, we felt it was an attractive candidate for regulating entry into the neural lineage.

Murine ES cells default to primitive neural stem cells (pNSCs) when plated at clonal density in minimal media. Using this neural colony forming assay on ES lines that enabled manipulation of Oct4 levels (Niwa et al., 2000), we showed that 50-60% of the Oct4 levels found in WT ES cells were most conducive to neural induction. The highest frequency of pNSC formation, 8.5%, was observed when Oct4 levels were kept in this range prior to the assay. This contrasted with a sphere forming frequency of 0.5% in the WT control group. Regarding the underlying mechanism, increased frequency of sphere formation is not explained by enhanced cell viability at the 50-60% Oct4 level, nor by diminished differentiation within the starting population of ES cells. Similarly, while we found that a basal level of Oct4 was required for proliferation, manipulating Oct4 levels above this threshold did not effect proliferation.

Given this, we propose that a pluripotent ES cell, expressing 50-60% of the Oct4 that WT ES cells do, possesses a molecular environment conducive to entry into the neural lineage. These results support the idea of Oct4 as a master regulator, suggesting that during gastrulation, cells expressing intermediate Oct4 levels in the epiblast may be primed to transition to neuroectoderm.
Effect of glycogen synthase kinase-3 beta (GSK-3ß) inhibition on human haematopoietic progenitor cells from cord blood.

J.P.Leek, S.A.Boxall, A.F.Markham, E.A. de Wynter

Leeds Institute for Molecular Medicine, JIF Building, St James’s University Hospital, Leeds LS9 7TF

The Wnt signalling pathway is closely associated with stem cell function and possibly progenitor cell commitment. In the absence of Wnt signals, cytoplasmic β-catenin is phosphorylated by glycogen synthase kinase -3 beta (GSK-3ß) and targeted for proteosomal degradation. Binding of Wnt ligands, results in the inhibition of GSK-3ß-mediated phosphorylation of β-catenin. We have examined the effect of GSK-3ß inhibition on haemopoietic progenitor cells, using three different GSK-3ß inhibitors. CD34+ cells were isolated from human cord blood and cultured in serum-free media supplemented with stem cell factor (SCF), Flt3-ligand (FL) and thrombopoietin (TPO), in the presence or absence of a GSK-3ß inhibitor. Cell proliferation was observed in all cultures. However, by day 8, the cell numbers in inhibitor treated cultures, were about 50% of the control cell numbers. The reduction in cell numbers was not due to apoptosis but did correlate with a lower proportion of actively cycling cells in the GSK-3ß treated cultures. Next, we examined the clonogenic progenitor cell content in all cultures using standard clonogenic assays. When 2 x 10³ cells were plated in the progenitor cell assay, there was little difference in the numbers of granulocyte-macrophage colonies (CFU-GM) generated from either the inhibitor treated or the untreated cultures. In contrast, there was a significant increase in the erythroid colony (BFU-E) numbers, from cultures treated with the GSK-3ß inhibitors (p<0.02 - 0.05). Morphological examination of the inhibitor treated cells revealed a difference in the proportion of blasts; 65% inhibitor treated, versus 36% among the controls. There was a partial block in differentiation to mature erythroid cells as determined by Glycophorin A staining. Inhibitor treated cultures consistently exhibited a lower percentage of cells expressing Glycophorin A.

We conclude that the GSK-3ß inhibitors used in this study exert an effect on the CD34+ cells by generating increased numbers of erythroid progenitors and partially blocking erythroid differentiation.
Gabriela Durcova-Hills

Abstract P15

The role of c-myc in the epigenetic reprogramming of germ cells into pluripotent stem cells

Gabriela Durcova-Hills, Naoki Myioshi and Azim M. Surani

The Wellcome Trust / Cancer Research UK Gurdon Institute, Tennis Court Road, CB2 1QN, Cambridge, UK

Germ cells are programmed to differentiate into functional gametes while retaining the information required for formation of new organism. However, under certain conditions this programming can be reverse in vitro when PGCs are re-programmed to pluripotent embryonic germ (EG) cells. Little is known about the PGC reprogramming to EG cells. In this study we are using a Representational Difference Analysis (RDA) screen identifying factors underlying the epigenetic reprogramming of PGCs into EG cells. We make cDNA libraries from 11.5 dpc PGCs and EG cells that were subjected to RDA. By combining differential screen and expression analysis we selected several candidates differentially expressed between PGC and EG cells. One candidate, c-myc is expressed in EG cells but not in PGCs. The role of c-myc in the reprogramming is further studied. This work provides the basis for the understanding the molecular mechanism underlying the epigenetic reprogramming of differentiated PGCs into pluripotent EG cells.
Nucleostemin expression in normal epidermis and in cancer

Dvořánková B.1,2, Lacina L.1,2,3, Smetana K. Jr1,2, Kaltner H.4, Lensch M.4, Gabius H.-J.4, André S.4

Charles University, 1st and 2nd Medical Faculty, 1Institute of Anatomy; 2Centre of Cell Therapy and Tissue Repair; 3Department of Dermatovenerology, Prague, Czech Republic 4Ludwig-Maximilians-University, Faculty of Veterinary Medicine, Institute of Physiological Chemistry, Munich, Germany

Adult tissue stem cells (TSC) play a key role in self-renewal and regeneration of tissues. At present, no reliable marker has yet been defined for this cell type. In this presentation we focus on evaluating expression of nucleostemin (Ncls) for this purpose. It is expressed in nucleoli of stem cells of different nature and related malignancies. However, no data on Ncls expression in squamous epithelia are available. Therefore, we monitored occurrence of Ncls in sections of normal human epidermis and in basal cell carcinoma. Furthermore, we tested cultured human keratinocytes and cells from the FaDu line originating from a squamous cell carcinoma. Expression was compared with expression of adhesion/growth-regulatory galectin-1 and with markers of proliferation and differentiation such as keratins.

In situ, Ncls was expressed in cells of the basal layer and the spinous layer. Presence in basal cell carcinoma was significantly higher than in normal epidermis. Normal cultured keratinocytes prepared from hair follicle cocultured with feeder cells presented in nucleoli with coexpression of galectin-1 akin to the situation in tumor cells.

In conclusion, while nucleostemin expression is not an exclusive marker of epidermal stem cells in situ, its expression characterizes a certain distinct functional status of cultured cells in vitro, where the selective coexpression of Ncls with Gal-1 suggests a low differentiation status of cells prepared from hair follicles.

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Trine Fink

Abstract P17

Hypoxic treatment inhibits insulin-induced chondrogenesis of ATDC5 cells despite upregulation of DEC1.

Trine Fink, Chen Li, Peter Ebbesen & Vladimir Zachar

Laboratory for Stem Cell Research, Aalborg University, Aalborg, Denmark.

In vivo chondrogenesis progresses in an environment where the supply of oxygen is limited. Under these conditions, the transcription factor hypoxia inducible factor 1, HIF-1, plays a regulatory role, possibly mediated through the transcription factor DEC1. We have analyzed the effect of hypoxia (1% oxygen) alone and in combination with insulin on the chondrogenic differentiation of the mouse embryonic stem cell line ATDC5. We found that the transcriptional activation of DEC1 was invariably enhanced by the hypoxic exposure. Paradoxically, while hypoxic treatment alone induced early chondrogenesis as evidenced by enhanced expression of aggrecan and collagen II, hypoxic incubation of insulin-treated cells delayed and suppressed insulin-mediated early chondrogenesis and almost completely blocked hypertrophic differentiation.
Anemia is observed in up to 60% of patients with cancer. PBI-1402 is an orally active low molecular weight synthetic compound which is currently in a phase Ib/II clinical trial in patients with anemia associated with cancer and/or chemotherapy. Clinical Phase I study showed that PBI-1402 is devoid of significant side effects in healthy volunteers. In addition, these results showed a significant increase (100%, p < 0.0001) of relative and absolute reticulocyte count in healthy volunteers after 21 days of oral treatment compared to placebo. PBI-1402 stimulates the in vitro/ex vivo proliferation and maturation of human hematopoietic progenitors (erythroid and myeloid populations) with an activity comparable to EPO. An additive effect on proliferation is observed in combination with EPO. Furthermore, in a human bone marrow colony formation assay, PBI-1402 enhances the differentiation of pluripotent stem cells: CFU-GEMM, CFU-GM with a predominant effect on BFU-E. Preclinical results demonstrated that PBI-1402 has an immunorestorative effect in myeloablative therapy or radiation induced by cytotoxic drugs and in anemia induced by phenylhydrazine. The effect of PBI-1402 results in an increase in erythrocytes into the systemic circulation. In myeloblated mice that received a syngeneic bone marrow transplant after lethal irradiation, oral treatment with PBI-1402 resulted in a significant increase in recovery of hemoglobin and peripheral erythrocyte and platelet count. In addition, PBI-1402 increases the number of bone marrow erythroid (CFU-E) cells and the more primitive Sca1+ progenitors. PBI-1402 is targeted as an orally active erythropoietin mimetic for the treatment of anemia.
Identification and Characterization of 14-3-3 sigma and Keratin 5 Expressing Adult Stem Cells

Medina A, Kilani RT and Ghahary Aziz

2660 Oak St., Vancouver, BC, Canada, V6H 3Z6

Introduction and Rationale: It is well established that upon any kind of dermal injury, epithelial cells of epidermis as well as those of hair follicles and other appendages become activated, and migrate toward the wound surface to form the epidermal layer of the newly healed wounds. However, this may not be the case for the full thickness (no appendages) large wounds, which take months for epithelial cells to migrate and epithelialize the wound area. As such, there must be another source from which epithelial cells originate and participate in wound epithelialization. Although, there is some evidence that bone marrow-derived stem cells can trans-differentiate into fibroblast-like cells known as fibrocytes, there is only indirect evidence that this might also be the case for epithelial cells. Here, we therefore, asked the question of whether PBMC have capacity to trans-differentiate into keratinocyte like cells (KLC). To address this question, we have cultured circulating PBMC and evaluate the expression of epithelial cell markers such 14-3-3 s and keratin 5 (K5).

Method: To evaluate the capacity of circulating adult stem cells to trans-differentiate into KLC, peripheral blood mononuclear cells (PBMC) were isolated from 50 mL of blood taken from healthy volunteers. PBMC were then cultured in test medium consisting of 49% keratinocyte serum free medium supplemented with EGF and pituitary extract, 49% DMEM and 2% FBS for different durations. Control cultures were human dermal fibroblasts (negative control) and keratinocytes (positive control) were also cultured in this medium. All cells were then examined for expression of two epithelial markers: KDAF and keratin 5.

Results: The results of this study revealed that KDAF used as an epithelial cell marker is expressed at the level of mRNA and protein in keratinocytes only, but not fibroblasts and immune cells using Northern and western blot analysis, respectively. This finding has been confirmed by immunocytochemistry and immunofluorescence microscopy showing that both KDAF and keratin 5 are strongly expressed only by keratinocytes, not fibroblasts. On day 1 of culture, PBMCs showed a distinct lymphatic cell morphology which was negative for both of these markers. However, over time the morphology of these cells changed dramatically to very closely resemble that of cultured keratinocytes. Concurrent with morphological changes, PBMCs became positive for KDAF as early as day 7 and remained high up to day 21 examined. However, as K5 is an intermediate differentiation marker, its expression became positive on day 21.

Conclusion: These results collectively suggest that circulating, bone marrow-derived stem cells, would trans-differentiate into KDAF and K5 expressing KLC.

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Expression of GAGE cancer/testis antigens in tumorigenic human mesenchymal stem cells

Morten F. Gjerstorff, Jorge S. Burns, Lene E. Johansen, Ole Nielsen, Moustapha Kassem and Henrik J. Ditzel.

Medical Biotechnology Center, University of Southern Denmark, Odense, Denmark; Department of Endocrinology and Metabolism, Department of Pathology, and Department of Medicine, Odense University Hospital, Odense, Denmark.

The hMSC-TERT20 cell line is derived from hMSC that had been transduced with the telomerase hTERT gene to compensate for the lack of endogenous telomerase activity in vitro and thereby increased the proliferative lifespan. Early cultures of hMSC-TERT20 cells (passage 30) were able to form bone when implanted in immunodeficient mice. However, late passage hMSC-TERT20 cells (passage 56) were surprisingly tumorigenic. This has provided a new model to study the stem cell hypothesis for cancer.

We have used the cell line to study changes in cancer testis antigen (CTA) expression associated with gain of tumorigenicity of hMSC-TERT20. We found that CTAs MAGE-A1, -A3, -A4 and SSX-1-9 were expressed in both normal and tumorigenic hMSC-TERT20, while only GAGE was significantly up-regulated in tumorigenic hMSC-TERT20 compared to the normal hMSC-TERT20.

To study the mechanism of induction of GAGE gene expression in hMSC-TERT20 we examined the effect of the demethylation agent 5-Aza-2'-Deoxycytidine and the histone deacetylase inhibitor Trichostatin A on GAGE expression. Trichostatin A treatment resulted in a large increase in GAGE expression, while only a small up-regulation was obtained by 5-Aza-2'-Deoxycytidine treatment, indicating that histone acetylation may contribute to induction of GAGE expression in hMSC-TERT20.

Analysis of single cell clones derived from tumorigenic hMSC-TERT20 exhibited different tumorigenic phenotypes, and we also found significant variation in the expression level of GAGE proteins. Four clones (BC8, BD6, CE8, BD11) exhibited strong expression of GAGE, while the BB3 clone was only weakly positive and the DB9 clone was negative. However, we observed no correlation between GAGE expression and proliferation rate, contact inhibition, anchorage-independent growth, serum dependence, xenograft tumorigenicity, or spheroid growth. Our results suggest that GAGE expression correlates with a tumorigenic phenotype, but is not needed for maintenance of tumorigenicity.
Calreticulin is essential for cardiac differentiation of embryonic stem cells.

Jody Groenendyk, Virginie Martin, Michel Puceat, Marek Michalak

Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada and INSERM-U421/I-Stem, Evry, France.

Calreticulin is an ubiquitously expressed calcium binding chaperone of the endoplasmic reticulum (ER). Calreticulin deficiency in mice is lethal in utero due to defects in heart development and function. Calreticulin-deficient embryonic stem (ES) cells can not be efficiently differentiated into cardiomyocytes and show impaired myofibrillogenesis. Cardiac differentiation of calreticulin-deficient cells can be restored by expression of full length calreticulin or calreticulin domain involved in Ca buffering in the ER. This indicates that ER Ca stores may play an important role in cardiac differentiation of ES cells. Molecular analysis of calreticulin-deficient ES-cell revealed that they have impaired ER-dependent Ca homeostasis. This, in turn, affects Ca-dependent transcriptional processes. Immunocytochemistry, Western blot and transcriptional analysis revealed that in calreticulin-deficient cells both MEF2C and NF-AT transcription factors are not efficiently translocated to the nucleus. In this study we show that this is due to a low or no activity of Ca-dependent phosphates calcineurin and to a reduced activity of MAPK signaling pathway in calreticulin-deficient ES cells. We concluded that calreticulin and Ca buffering in the ER are essential for normal cardiac differentiation of ES cells. Both calcineurin- and MAPK–dependent pathways are affected by ER Ca homeostasis.
PBI-1402 is a non-toxic, well-defined low molecular weight synthetic hematopoietic differentiation and growth stimulant. PBI-1402 promotes the proliferation of hematopoietic progenitors (myeloid and erythroid populations) yielding a biological efficacy comparable to G-CSF, GM-CSF, IL-3, SCF, TPO and EPO in in vitro human bone marrow cell proliferation. An additive effect on proliferation is observed when PBI-1402 is combined with G-CSF, GM-CSF, IL-3, TPO and EPO. Furthermore, in a human bone marrow colony formation assay, PBI-1402 enhances the differentiation of pluripotent stem cells: CFU-GEMM, CFU-GM with a predominant effect on BFU-E. PBI-1402 exerts its activity via a different mechanism of action than EPO and at an earlier stage on more immature hematopoietic progenitors. PBI-1402 is targeted as an adjunct to cancer chemo/radiotherapy, bone marrow transplantation and diseases involving anemia and neutropenia.
Compromised immune responses and decreased vaccine efficacy are age-related perturbations that result from diminishing humoral immunity. The effects of aging on the immune system are widespread and extend from hematopoietic stem cells (HSCs) and lymphoid precursors. Studies in our laboratory and many others have led to the idea that a major cause of “immunological aging” results from the failure of aged HSCs to give rise to a fully functional B-cell compartment.

We hypothesize that reduced B-cell production in the bone marrow (BM) results in the selective accumulation of long-lived, antigen experienced cell in the periphery. We believe that eventually these cells dominate the peripheral repertoire and the immune system is forced to rely upon them to mount a response to newly encountered pathogens, such as West Nile Virus, SARS, influenza, etc. In order to address the cause of decreased B-cell production in the bone marrow, we have developed unique and innovative tools to approach our hypothesis. These include a transgenic system in mice that allows quantification of aging, as well as the generation of conditionally and reversibly transformed long-term repopulating hematopoietic stem cell (LT-HSC) lines from young and aged mice. These cell lines appear to retain the defects identified in their precursors, i.e. the aged HSC line is unable to reconstitute the B cell compartment.
Embryonic stem (ES) cells are characterised by their ability to self-renew, whilst retaining their ability to differentiate into all the cell types of the embryo. Mouse ES cells have been studied for over 25 years, yet until recently only a few genes had been identified as having specific or critical roles in ES cells. What is understood of the mechanisms of self-renewal and pluripotency is that a key interplay between endogenous transcription factors (such as oct-4, sox2 and nanog) and exogenous signalling molecules (such as LIF and BMPs) is required to maintain the undifferentiated stem cell state.

The POU class V transcription factor oct-4 is an essential marker of pluripotency and serves as both an activator and repressor of transcription. Prolonged decrease of oct-4 levels leads to trophectodermal lineage differentiation, with concomitant up-regulation of markers cdx2, eomes and hand1. Inducible oct-4 perturbation combined with microarray profiling of an acute time-course, has allowed identification of gene profiles which precede and converge with trophectodermal marker up-regulation. Characterisation of a subset of these genes, which are specifically enriched in ES cells compared to other self-renewing populations (neural and trophoblast stem cells), will add to a more comprehensive understanding of the ES transcriptome.

Expression of oct-4 is not sufficient to drive self-renewal in the absence of cytokine signalling. mES cells are routinely cultured in the cytokine LIF and through downstream effectors including STAT3, maintain the undifferentiated state in vitro. However, elevated nanog expression has been shown to bypass the requirement for exogenous signalling factors. Performing a microarray meta-analysis of nanog over-expressing and WT cells it has been possible to identify genes downstream of LIF which have a role in mES cell self-renewal.

Kyle Howerton

Abstract P25

Chondrogenic Differentiation of Mouse Derived Adult Germ Cells

Kyle Howerton, Jane Pham, Francisco Silva, Fari Izadyar, Carl Javier, Francis Pau, Joel Marh, Natasha Slepko, and Chauncey Sayre

PrimeCell Therapeutics LLC, 213 Technology Drive, Irvine, CA 92618, USA.

We have reported previously that post-natal adult germline stem cells selectively isolated from a transgenic mouse model (OG-2 mice with a germ line-specific Oct-4 promoter-driven GFP reporter gene) can be transformed into pluripotent adult cells (AGCs) by culture-induced reprogramming. These mouse pluripotent AGCs display pluripotent markers and characteristics that are similar, but not identical, to mouse embryonic stem cells (mESCs). Both mESCs and mAGCs form embryoid bodies that can be directed to differentiate into neural cells and cardiomyocytes. However, mESCs, but not mAGCs, form teratomas after in vivo transplantation into SCID mice. Thus, mAGCs have the potential to become a valuable cell source for cell-replacement therapy. Arthritis and autoimmune diseases frequently involve chondrocyte degeneration, and chondrogenic cell replacement therapy has been studied as an alternative treatment. We report here that mouse AGCs can be directed to differentiate into phenotypes that exhibit chondrocyte characteristics. Mouse AGCs were subjected to chondrogenic differentiation, for a total of 25 days, using a combination of low serum concentration (1%) and TGFβIII. Following this treatment, mAGCs showed typical morphology associated with chondrogenesis. This was verified by the presence of cartilage associated proteoglycans indicated by positively stained Alcian blue and Aggrecan cells. Next we verified gene expression with RT-PCR and found up-regulation for two chondrogenic transcription factors, Sox9 and Cbfa1, as well as two chondrocyte associated collagen genes (collagen IIa1, a chondrocyte marker; collagen Xa1, a hypertrophic chondrocyte marker). These results demonstrate that mAGCs have the potential to undergo chondrogenesis when cultured in the appropriate conditions.
Abstract P26

Mediation of apoptosis and proliferation of human embryonic stem cells by Sphingosine-1-phosphate.

Katie Inniss and Harry Moore.

Centre for Stem Cell Biology, University of Sheffield, Western Bank, Sheffield, S10 2TN, UK.

Human embryonic stem cells (hESCs) replicate by the process of self-renewal while maintaining pluripotency. This process is poorly understood and is thought to be regulated by several different pathways. Sphingosine-1-phosphate (S1P) is a bioactive lipid which plays a role in the regulation of cell fate in many cell types. S1P has been implicated in a diverse range of biological processes including cell survival, proliferation, differentiation, migration and apoptosis. In this study we address the apoptotic and proliferative response of hESCs to exogenous S1P application and demonstrate a role for lysophospholipid signalling in stem cell maintenance. S1P receptors were localised by immunofluorescence on several lines of hESCs (Shef 1-6), and their presence verified by RT-PCR and western blotting. Dual staining of S1P receptors with the stem cell marker Tra-1-60 confirmed the presence of receptors on pluripotent cells and therefore suggested that the S1P pathway was active in hESCs. Treatment with S1P supplemented media significantly reduced the level of apoptosis witnessed in hESC cultures and was also found to stimulate proliferation. These results indicate a role for S1P in the self-renewal of hESCs and further support the use of S1P in defined media by identifying its ability to aid survival and proliferation of cell lines. Further investigations are in progress to determine a mode of action for this sphingolipid. ERK1/2 is a known downstream target of S1P in various cell types, with S1P stimulating activation of the ERK1/2 pathway. To date, ERK1/2 activation in hESCs is not fully comprehended, although it has been strongly suggested ERK activation is required in the maintenance of pluripotency, this pathway could also prove to be crucial in the self-renewal of hESCs by S1P.
Germ line stem cells are the only stem cells that can be converted to pluripotent stem cells through culture-induced reprogramming. Numerous studies have shown that human primordial germ cells (PGC) collected from 5-9 weeks human fetuses can be reprogrammed into pluripotent embryonic germ (EG) cells in culture using a mixture of growth factors. While human EG cells derived from PGCs, like that of human embryonic stem (ES) cells, express all the pluripotent markers, exhibit the potential to differentiate into phenotypes derived from all three germ layers, all existing EG lines have limited self-renewing capacity and stop proliferation after a few passages. In this study we investigated the possibility of reprogramming of human fetal gonocytes collected from fetuses between 14-20 weeks of gestation. Fetal gonocytes were cultured on STO feeder cells in a serum-free reprogramming medium (PM-10 medium™) containing a mixture of growth factors. After two weeks of culture, small colonies were manually transferred to a fresh STO feeder. Following 2-3 passages outgrowths appeared and flat colonies were formed. Similar to the existing EG cell lines, our EG cells exhibited pluripotent markers including SSEA-4, TRA-1-60, TRA-1-81, Alkaline Phosphatase, Oct-4 and Nanog and differentiated into cells of all three germ layers. However, the morphology of our EG cell colonies are, in contrast to existing cell lines, similar to human, rather than mouse, ES colonies. In addition, our EG cell lines exhibited high telomerase activity after 10 passages and maintained their self-renewal capacity. As human EG cells do not generate teratomas after transplantation into the immune deficient recipients, our EG lines generated from human fetal gonocytes could become a novel and safe alternative pluripotent cell source for application in regenerative medicine.
Most tumors are clonal in origin; they arise from a single cell that has acquired the capacity to proliferate and give rise to the heterogeneous populations of cells comprising the tumor. As such, this tumor cell-of-origin may be thought of as a “cancer stem cell” (CSC). Several recent studies performed on a variety of tumor types have demonstrated the presence of a subpopulation of tumor cells with a high innate capacity for tumor propagation upon transplantation, supporting the idea of CSCs. It is likely that dysregulation of the pathways involved in normal stem cell survival and self-renewal may underlie CSC proliferation. We examined the expression of various growth factor receptors on adult neural stem cells and putative adult lung stem cells in vivo and assessed their effects on cell proliferation and self-renewal in vitro. We found that adult neural stem cells express the PDGFRa and respond to PDGF signaling both in vitro and in vivo. Overexpression of PDGF ligands and their cognate receptors occurs with equal frequency in the majority of gliomas of all grades, suggesting it plays a role in tumor initiation. Strikingly, infusion of the PDGF ligand into the lateral ventricle results in the induction of atypical hyperplasias resembling low- to intermediate-grade human gliomas. Interestingly, our preliminary findings in the lung suggest that the receptors for several growth factors regulating lung development are also expressed on adult lung stem cells and on lung tumors in a mouse lung adenocarcinoma model. Our analysis of the effects of these growth factors on adult lung stem cells are ongoing.
In vitro embryonic stem cell differentiation as a tool to study heparan sulphate in early developmental systems

Claire E Johnson¹, Graham Rushton¹, Gerdy ten Dam², Marios Stavridis³, Jeff Esko⁴, Toin van Kuppevelt², Austin Smith⁵, John Gallagher¹ and Catherine Merry⁶

¹CRUK Department of Medical Oncology, University of Manchester M20 4BX
²Dept. Biochemistry 280, Nijmegen Center for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, PO Box 9101, 6500 HB Nijmegen, The Netherlands
³Division of Cell and Developmental Biology, University of Dundee, DD1 5EH
⁴Department of Cellular and Molecular Medicine, Glycobiology Research and Training Center, University of California, San Diego, La Jolla, USA 92093
⁵MRC Centre of Stem Cell Biology, Institute for Stem Cell Research, University of Edinburgh, King's Buildings, West Mains Rd., Edinburgh, Scotland, UK
⁶School of Materials, Materials Science Centre, The University of Manchester, Manchester M1 7HS

Mouse embryonic stem (ES) cells provide a malleable experimental system in which to study the structural features and biological functions of heparan sulphate (HS) proteoglycans. HS is a complex polysaccharide with a hypervariable sulphated fine structure that is arranged in domains that are specified by pattern and degree of sulphation. HS is known to regulate growth factor signalling and morphogen gradients during embryonic development and in adult life, and HS null mice do not progress through early embryogenesis. Our work utilises a variety of mES cell lines, including HS-competent, HS-null (Ext1⁻/-) and HS-mutant lines to study the role of HS and HSPGs in both ES cell biology and early differentiation pathways. We find that HS is not required for the maintenance and self-renewal of the ES cell phenotype, however the cells’ ability to differentiate into the three germ layers, a key characteristic of the ES phenotype, is impaired in the absence of endogenous HS. This has been studied using several experimental models of differentiation, including use of embryoid bodies and monolayer neural induction. In the FGF-dependent neural differentiation experiment the Ext1⁻/- cells convert to neural precursor phenotype with extremely poor efficiency; this can be rescued with the addition of exogenous heparin, a close relative of HS. Previous studies using a wild-type ES line with GFP knocked into the neural precursor marker Sox-1 have proved extremely valuable in the characterisation of cell-type specific HS chains. Current experiments involve the generation of a Sox1-GFP reporter Ext1⁻/- ES line, followed by add-back experiments using HS oligosaccharides selected for their potential to augment specific growth factor signalling pathways. It is anticipated that this strategy will provide further understanding of HS-growth factor interactions in a biological setting.
Philip Jones

Abstract P30

A single type of progenitor cell maintains normal epidermis

Elisabeth Clayton 1, David P Doupé 1, Allon M. Klein 2, Douglas J Winton 3 Benjamin D Simons 2 and Philip H Jones 1

1 MRC Cancer Cell Unit, Hutchison-MRC Research Centre, Cambridge CB2 2XZ, UK
2 Cavendish Laboratory, University of Cambridge, Madingley Road Cambridge CB3 0HE, UK.
3 Cancer Research UK Department of Oncology, University of Cambridge, Cambridge Institute

According to the current model, adult epidermis is maintained by two discrete populations of progenitor cells. These comprise self-renewing stem cells and their progeny, known as transit amplifying (TA) cells, which go on to differentiate after several rounds of cell division1-3. We have used inducible genetic labelling to track the fate of a representative sample of progenitor cells at single cell resolution for a year in vivo. By analysis of clone size distributions we show that normal epidermis is maintained by only one type of progenitor cell which undergoes asymmetric division at a rate that ensures epidermal homeostasis. We challenged the predictive power of this new paradigm in epidermis rendered highly proliferative by retinoic acid treatment, and found that the model successfully predicted the observed clone size distribution. These results raise important questions on the role of stem cells in vivo and on the development of cancer.
Ken-ichiro Kamei

Abstract P31

An Integrated Microfluidic Platform for Screening hESC Culture Conditions

Ken-ichiro Kamei, Zeta Tak For Yu, Shuling Guo, Carol Suh, Runsheng Wang, Jenny Shu, Caius Radu, Owen N. Witte, and Hsian-Rong Tseng

700 Westwood Plaza, Box 9517770, Los Angeles, CA 90095, USA

Microfluidics, with its intrinsic advantages of sample/reagents economy, precise control over physical and chemical microenvironments, high throughput, scalability and digital controllability, is a prime operation platform for screening ideal hESC culture conditions. In contrast, the conventional hESC research is plagued by the use of macroscopic culture conditions, associated with several constrains, e.g., high sample/reagent consumption, poor precision to control and monitor the microenvironments of individual hESC colonies and the lack of integrated platforms for accurate phenotypic and functional measurements. In this study, three generations of microchip for hESC-culture (hESC-Chip) have been designed, fabricated and tested. These studies demonstrated that HSF1 hESC could be cultured in a stand-alone microfluidic device with a 99.9% reduction in the sample/reagent consumption. Moreover, implementation of fluorescence-and bioluminescence-based bioassays allowed the quantification of the growth rates of Chip-cultured hESC colonies carrying green fluorescent protein (GFP) and Firefly luciferase (Fluc). Finally, sequential immuno-stainings using pluripotency markers (alkaline phosphatase, Oct-4 and SSEA-4) were performed in situ to confirm the stemness of these colonies. Currently, we are developing new-generation hESC-Chips for fully automated hESC culture and assays. A typical hESC-Chip will contain 100 individually addressable culture chambers array in a 10 x 10 format. Critical hESC culture parameters such as temperature, humidity, pH, pO2 and flow rate of culture media will be continuously monitored and precisely control via a digital interface. The device will incorporate several phenotypic assays to characterize individual hESC colonies (i.e. real-time CCD imaging, on-chip immunocytochemistry and RT-PCR); additional functional assays will be gradually incorporated in subsequent generations. This platform enables an exponential increase of screening variables in search of hESC culture conditions with feeder-free and serum-free hESC culture conditions for hESC renewal and differentiation.
Jana Karbanova

Abstract P32

Characterization of human dental pulp stem cells

Jana Karbanova¹, Tomas Soukup¹, Martin Valis², Jaroslav Mokry¹, Jakub Suchanek³, Robert Pytlik⁴, Lenka Kucerova⁵, Dana Cizkova¹

¹Department of Histology and Embryology, Charles University in Prague, Faculty of Medicine in Hradec Kralove, Czech Republic
²Department of Neurology, Teaching Hospital in Hradec Kralove, Czech Republic
³Department of Stomatology, Teaching Hospital in Hradec Kralove, Czech Republic
⁴1st Department of Internal Medicine, 1st Medical Faculty of Charles University, Prague, Czech Republic
⁵Department of Clinical Genetics, Teaching Hospital in Hradec Kralove, Czech Republic

Mesenchymal stem cells (MSCs) represent promising source of cells for replacement of damaged tissues. We isolated dental pulp stem cells (DPSCs) from extracted third molars and cultured in a serum-low-content medium supplemented with growth factors PDGF-BB and EGF. These cells shared the similar antigenic, morphological and differentiation potential characteristics with MSCs. DPSCs were characterized as a cell population that was STRO-1⁺, CD29⁺, CD44⁺, Thy-1⁺, HLA I⁺, CD49a,c,d,e low, CD11b,c low, c-kit low, CD34 - , CD45 - and HLA II -.

Isolated stem cells were expanded over the 50 population doublings (PD) and during the whole culture DPSCs showed stable karyotype. DNA analysis demonstrated that 42% cells were in SG₂ phase. Immunocytochemical analysis confirmed expression of stem cells associated markers including telomerase (hTERT), CXCR4, nucleostemin, nestin, β₁-integrins, and weak positivity for VEGFR2, Bcrp1 and MDR1 and negativity for neural, vascular and hepatic cell markers. When placed in osteogenic media DPSCs were found to differentiate in osteonectin and procollagen type I⁺ osteoblasts. In micromass cultures cells formed osteogenic pellets rich in collagen with depositions of calcium. Chondroblasts were generated in chondrogenic pellets in TGF-β1 supplemented media and produced extracellular matrix typical for cartilaginous tissue. Endothelial cell differentiation of DPSCs was induced in serum-free media containing VEGF. DPSCs there differentiated and exhibited morphology and phenotype characteristic for endothelial cells. When the cells were placed in medium for NSCs supplemented with serum, they formed spheroids and exhibited markers of immature neural cells (nestin, A2B5, NG2, RC2, SSEA-1). Exclusion of serum from media facilitated cell differentiation and expression of neuronal and glial markers.

Our results demonstrate that isolated DPSCs are homogenous self-renewing population of cytopogenetically stable multipotent cells that are able to generate bone, cartilage, vascular and neural cells. This indicates that DPSCs possess broader differentiation potential then was previously published.

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Phillip Karpowicz

Abstract P33

DNA is Partitioned Asymmetrically in the Germ Stem Cells of the Drosophila ovary

Phillip Karpowicz¹, Milena Pellikka², Dorothea Godt², Ulrich Tepass², Derek van der Kooy¹

¹ Department of Medical Genetics and Microbiology; ² Department of Zoology, University of Toronto, Toronto, Canada

The Immortal Strand Hypothesis proposes that asymmetrically dividing stem cells segregate chromosomes non-randomly to retain ancestral DNA templates. This asymmetric segregation of DNA was investigated in germline stem cells of the Drosophila ovary, which divide only asymmetrically generating a new stem cell and a differentiating cystoblast. Asymmetric partitioning of DNA occurs in germline stem cells in vivo. When germ stem cells are forced to differentiate by overexpression of Bag of Marbles, no asymmetric partitioning of DNA is observed between germ cell progenitors, showing that this process only occurs in stem cells and not their progeny. Moreover when asymmetric division is disrupted the uneven partitioning of DNA does not occur. Ectopic expression of Decapentaplegic, a BMP family morphogen that causes both daughter cells to adopt stem cell identity, abolishes this asymmetric partitioning of DNA. Similar disruption is observed in Bag of Marbles mutants whose inability to differentiate into cystoblasts also abolishes the asymmetric partitioning of DNA. These results support chromosome cosegregation in germ stem cells in vivo and show that cosegregation is coupled to mechanisms specifying cellular differentiation via asymmetric cell division.
Jayne Keifer

Abstract P34

A Novel Immunogenicity Assay Using the Affibody Ligand Can Overcome Inherent Control Challenges.

Jayne A. Keifer, Kristen Mark, Gwendolyn M. Wise-Blackman, Ana T. Menendez

160 Cardinal Health Way, Morrisville, NC 27560

Biopharmaceutical therapies have the potential to induce immune responses that can cause serious side effects or lead to a loss of therapeutic efficacy. Regulatory agencies are placing heavier scrutiny on biological therapeutics for identification of antibody responses prior to the initiation of clinical trials. The inherent difficulties of finding positive human controls to cell therapies and biotherapeutic agents makes immunogenicity assay development challenging. The ability to validate a single method that recognizes immune responses of differing species is conducive for testing and identifying positively reacting serums prior to and during clinical trials and is essential for the progression of biotherapeutic medicine. In order to address this issue, our laboratory has developed a novel assay method that can accommodate both multi-species controls and identify antibody responses to a biological therapeutic in human samples using the Affibody technology. With this method we can identify reactive serum derived from human, non-human primate or rodent with the Affibody affinity ligand that will recognize diverse species. This screening assay utilizes flow cytometry methods to detect antibody responses against cell and biotherapeutic therapies allowing us to perform quasi-quantitative immunogenicity assays using just a single method. The Affibody technology used in this method overcomes the lack of appropriate controls or complications associated with the multi-species nature of controls used in immunogenicity assays. Furthermore, this method can be a powerful tool to the scientific community in the development of biotherapeutic medicines because it can provide subject immune status during both pre-clinical and clinical trials.
Immature dental pulp stem cells expressing OCT-4 and other embryonic stem cells markers showed dense homing and differentiation in immunocompetent mouse

Kerkis, I 1, Fonseca, SAS 2, Santos EJC 1, Lavagnolli, TMC 1,2, Maranduba, CMC 1, Sant’Anna OABE 1, Serafim RC 2, Abdelmassih, S 2, Monteiro BG 1, Pereira, LV 2, Cerruti, H 2, Kerkis, A 2

1 Laboratório de Genética, Instituto Butantan, São Paulo, SP, Brasil. 2Clínica e Centro de Pesquisa em Reprodução Humana “Roger Abdelmassih”, São Paulo, SP, Brasil.

We isolated a population of human immature dental pulp stem cells (IDPSC), which express embryonic stem cell markers, such as Oct-4 and Nanog, as well as several other mesenchymal stem cell markers, during at least 25 passages with normal karyotype and rate of expansion characteristic of stem cells. The expression of these markers was maintained in sub-clones that were obtained from those cells. Moreover, IDPSC can be induced to undergo uniform differentiation in vitro, under chemically defined culture conditions, into smooth and skeletal muscles, neurons, cartilage and bone. We produced anti-IDPSC antibody and demonstrated the homing capacity of IDPSC after their in vivo transplantation into immunocompetent mice. The presence of IDPCS within the mouse organs was confirmed by PCR, using specific primer for human Gauche disease gene. IDPSC showed dense engraftment and differentiation in various tissues such as liver, lung, spleen and brain, which were analyzed by immunofluorescence using tissue specific antibodies. Moreover, it seems that differentiation of IDPSC in vitro and in vivo could occur through two mechanisms: fusion and differentiation. The relative ease of recovery and the expression profiles of various markers justify further exploration of these dental pulp stem cells for clinical therapies.
Laura Kerosuo

Abstract P36

c-myc increases self-renewal in Neural Stem Cells

Kerosuo Laura, Piltti Katja, Fox Heli, Angers-loustau Alexandre, Häyry Valtteri, Sariola Hannu & Wartiovaara Kirmo

Department of Developmental Biology, Biomedicum, University of Helsinki, Finland

Mammalian neurogenesis takes place mostly during embryonic development and early childhood, but some renewing activity is maintained also through adulthood. Stem cell maintenance is a consequence of various intrinsic and extrinsic cues that are provided and regulated by the cells of the stem cell niche. During development or in case of an injury, self-renewing tissues are programmed to react to the changing demands of the numbers of produced cells. In order to permit dynamic control of the stem cell pool the self-renewing cells need strategies that can be utilized in case the rate of regeneration aught to be changed. We have approached the question of self-renewal maintenance by overexpressing oncogenes in neural stem cells (NSCs) and thereby trying to reveal the role of these genes in controlling the stem cell capacity. Here we present results on the affects of transgenic retroviral overexpression of the oncogene c-myc in embryonic E13-16 NSCs in vitro. When NSCs are cultured as neurospheres in EGF / FGF containing medium in the absence of serum, over-expression of c-myc increases stemness of the cells as measured by several different cell biologic standpoints: c-myc doubles the cell proliferation rate and changes the regulation of the stem cell pool by dramatically increasing the number of self-renewing cells in the neurosphere niche. Also, a very small subpopulation of the myc overexpressing cells, 2,5%, maintain self-renewal and refuse to exit cell cycle despite the cell culture conditions that provoke differentiation (2% FCS). The majority (97.5%) of the myc-overexpressing cells, on the other hand, read the differentiation cues in the same way as WT-cells: they exit the cell cycle within 48 hours and start expressing differentiated morphology as well as neuron and astrocyte specific proteins such as TUJ and GFAP. However, the morphology of these differentiated myc-cells looks disturbed as compared with WT cultures. Interestingly, majority of the progeny of the 2,5% of the cells that maintained self-renewal are again able to exit cell cycle and differentiate when recultured. We have also used flow cytometry in order to measure stem cell capacity by quantifying changes in the expression of stemness-associated antigens of neural progenitors. C-myc increases the expression of Bmi-1 and nestin in neurospheres and both markers are lost during differentiation. In conclusion we propose that c-myc plays a role in controlling the balance of self-renewal of NSCs in the neurosphere niche.
Chul Geun Kim

Abstract P37

Profiling of Differentially Expressed Genes in Human Stem Cells

Jong Joo Lee, Jinson Jeon, Hyen Seok Heo, Ho chul Kang, Byung-Rok Do, Chul Geun Kim

Department of Life Science, Hanyang University, Seoul 133-791, Korea

Stem cells are unique cell populations with the ability to undergo both self-renewal and differentiation, although a wide variety of adult stem cells as well as embryonic stem cells have been identified and stem cell plasticity has recently been reported. To identify genes implicated in the control of the stem cell state as well as the characteristics of each stem cell line, we analyzed the expression profiles of genes in human embryonic, hematopoietic (CD34+ and CD133+), and mesenchymal stem cells using cDNA microarrays, and identified genes that were differentially expressed in specific stem cell populations. In particular we were able to identify potential hESC signature-like genes that encode transcription factors (TFAP2C and MYCN), an RNA binding protein (IMP-3), and a functionally uncharacterized protein (MAGEA4). The overlapping sets of 22 up-regulated and 141 downregulated genes identified in this study of three human stem cell types may also provide insight into the developmental mechanisms common to all human stem cells. Furthermore, our comprehensive analyses of gene expression profiles in various adult stem cells may help to identify the genetic pathways involved in self-renewal as well as in multi-lineage specific differentiation.
Stem cells have potential to generate diverse lineage of cells and to renew themselves by maintaining proliferation properties. Mesenchymal stem cells have potential to differentiate into osteoblasts, chondrocytes, myoblasts, fibroblasts, tenoblasts, and adipocytes. Stem cell population in periphery has been shown to be increased when stress conditions occur in the body. This increase may reflect the need for more stem cell proliferation and at the same time more generation of stem cell-derived functional cells to recover or combat the stress. Therefore, we reasoned that inflammatory conditions may also affect stem cell proliferation and/or functional cell differentiation from stem cells. As an example, we examined effects of interleukin 17, a cytokine increased in rheumatoid arthritis, on human mesenchymal stem cells obtained from bone marrow. Interleukin 17 stimulated osteoblastic differentiation of mesenchymal stem cells. The osteogenesis stimulatory effect was confirmed by both alkaline phosphatase staining and von Kossa mineralization test. Interleukin 17 also increased chemotaxis of mesenchymal stem cells and induced expression of some chemokines and chemokine receptors. Taken together, interleukin 17 may stimulate osteogenesis of mesenchymal stem cells through chemokine system regulation under inflammatory conditions to prevent aggravation of the bone loss induced by certain inflammatory cytokines such as IL-1α and TNF-α.
Stem cells differentiate by expressing a broad range of genes in the early stages of development and shutting more and more down during their lineage differentiation. Controlling this process is essential for stem cell application in medicine or tissue engineering. Reversing it could even lead to a new way of creating pluripotent stem cells. One of the main influences on lineage differentiation is the stem cell niche. The Extracellular matrix (ECM) is a main component of the niche and by interacting with integrin receptors it can directly influence the stem cells and its lineage differentiation. The exact mechanism behind this however is still unknown. Here we present our findings in the investigation of the underlying mechanism behind the influence of the niche and especially integrin receptor signaling. We investigated integrin expression in MSCs and how integrin expression pattern is correlated to signaling and stem cell differentiation. Bone marrow derived mesenchymal stem cells (MSCs) were induced to differentiate into endothelial cell (EC) while cultured in the presence of different ECM proteins.
PBI-1402: An orally active hematopoietic stimulant

M. Lagraoui¹, B. Grouix¹, N. Julien¹, M. Asselin¹, D. Gaudreau¹, F. Sarra-Bournet¹, C. Penney¹, L. Gagnon¹, M.-J. Morin², G. Krosl², D.-C. Roy²

¹ ProMetic BioSciences Inc., Laval, Québec, Canada
² Hôpital Maisonneuve-Rosemont, Montréal, Québec, Canada

Hematopoiesis is regulated by an intricate network of transcription factors and other molecules that mediate proliferation and differentiation of stem cell progenitors into myeloid and erythroid lineages. PBI-1402 is a non-toxic, well-defined chemo- and radio-protective orally active small molecule with capacity to promote hematopoiesis. In vivo studies demonstrate that PBI-1402 has an immunorestorative effect in myeloablative therapy induced by cytotoxic drugs or radiation and in anemia induced by phenylhydrazine. In mice immunosuppressed by cytotoxic agents (cyclophosphamide, 5-fluorouracil and taxotere), PBI-1402 yields an increase in bone marrow and spleen cell count. In phenylhydrazine-induced anemia, PBI-1402 treated mice had an increased number of hematopoietic progenitor cells compared to the control mice. This increase was more pronounced in the erythroid lineage (BFU-E and CFU-E). In myeloablated mice that received a syngeneic bone marrow transplant after lethal irradiation, oral treatment with PBI-1402 resulted in a significant increase in peripheral erythrocyte count and hemoglobin concentration. In addition, PBI-1402 accelerates white blood cell and platelet recovery and also increased the more primitive Sca1+ progenitors. In conclusion, these results indicate that PBI-1402 stimulates the proliferation and differentiation of stem cells. Therefore, PBI-1402 may be useful for the treatment of anemia associated with chemotherapy, radiotherapy, bone marrow transplantation and cancer.
Sally Lansley

Abstract P41

Bone formation in malignant mesothelioma tumours is dependent on the transdifferentiation potential of cells of mesothelial origin

SM Lansley¹, CM Prêle², RG Searles¹, SE Herrick³, PJ Thompson¹, SE Mutsaers¹

¹Lung Institute of Western Australia, ²Telethon Institute for Child Health Research and Centre for Child Health Research, University of Western Australia, Perth, Western Australia & ³University of Manchester, UK.

It has been reported that serosal pathologies including pleural malignant mesothelioma (MM) show features of osteogenic tissue formation, although its origin is unknown. We hypothesize that cells of mesothelial origin can differentiate into other mesenchymal cell types and that the local conversion of mesothelial cells to osteoblast-like cells is responsible for the observed osteogenic tissue. To test this hypothesis, rat mesothelial cells were collected from the omentum and peritoneal fat pads of 6-8 week old male Lewis rats, human mesothelial cells obtained from pericardial fluid of patients and MM cells obtained from pleural effusions of mesothelioma patients. Mesothelial cells were characterized via FACS analysis to determine expression of the classic stem cell markers and rat mesothelial cells were also purified based on their expression of HBME-1. A functional assay of bone nodule formation was performed in vitro with cells maintained in either osteogenic medium (OM) containing β-glycerophosphate, ascorbic acid and dexamethasone or standard culture medium for 0-27 days. Mesothelial cells and MM cell lines cultured in OM expressed alkaline phosphatase, an early marker of the osteoblast phenotype, and formed mineralized bone-like nodules as demonstrated by von Kossa stain. All cell populations examined expressed mRNA and protein of several key osteoblast markers, including bone specific core binding factor alpha 1 (Cbfa1) by RT-PCR and Western blotting. Histological analysis of a human MM biopsy demonstrated multiple foci of mineralized osseous metaplasia with associated Cbfa1 immunostaining. In conclusion, we have strong evidence that normal and malignant mesothelial cells have the capacity to differentiate into cells of the osteoblast lineage further illustrating their multipotential nature. These results also provide evidence that mesothelial differentiation may be responsible for the bone formation observed in MM tumours.
Soo-Jin Lee

Abstract P42

Relation of Tumorigenic Activity and Sphere-forming Capability in Human Breast Stem Cell Lines

Soo-Jin Lee¹, ², Nam-Shik Ahn¹, ², Yong-Soon Lee¹, ², Kyung-Sun Kang¹, ²*

¹ Adult Stem Cell Research Center and ² Laboratory of Stem Cell and Tumor Biology, Department of Veterinary Public Health(BK21), College of Veterinary Medicine, Seoul National University, Seoul, Korea. kangpub@snu.ac.kr.

For long time, many researchers have studied cancer therapy. The therapy shrinks the tumor and appears to be successful, but then the tumor grows back. This phenomenon has been accounted for very small number of 'cancer stem cell', that is, the therapy killed the susceptible cells, but the resistant stem cells were remained. According to 'Cancer stem cell theory', cancer originates in either stem cell or their progeny through mutation or dysregulation of the process of self-renewal. Therefore, they share the stem cell properties, self-renewal and resistance to apoptosis.

In this study, we established and have maintained several human breast epithelial stem cell lines-M13SV1, M13SV1 R2, M13SV1 R2N1- by mammosphere culture. We show that size and number of mammospheres increased in higher tumorigenic cell line. These studies support that survival of the cancer stem cell has a close relation with tumorigenicity in suspension culture system.
Abstract P43

Reduction of Liver Fibrosis by Xenogeneic Human Umbilical Cord Blood and Adipose Tissue-derived MSC without Treatment of Immunosuppressant

Yeong-Geon Lee1,2, Sun Bo1,2, Yun-Hyeok Jeong1,2, Kyung-Hwan Roh1,2, Sang-Bum Park1,2, Sae-Rom Lee1,2, Jae-Woong Hwang1,2, Il-Seob Shin3, Yong-Soon Lee1,2 and Kyung-Sun Kang1,2*

1 Department of Veterinary Public Health and BK21 Program for Veterinary Science and 2 Adult Stem Cell Research Center, College of Veterinary Medicine, Seoul National University, Seoul 151-742 and 3 Stem Cell Research Institute, RNL BIO CO., Ltd., Seoul 153-768, South Korea

Liver fibrosis is the wound healing process to the various liver injury and is characterized by the continuous collagen deposition in the extracellular matrix (ECM). In this study, six week-old one hundred and five Sprague-Dawley rats were housed and ninety of them were induced to the liver fibrosis and cirrhosis using dimethylnitrosamine (DMN) at 3 consecutive day each week for 5 weeks. At that time of the symptoms regarded as liver disease was shown in the general examination and serological and histological test, DMN injection was stopped and human umbilical cord blood (UCB) and human adipose tissue (AD) derived MSC were injected intravenously. In the serum analysis, total protein (TP) and albumin (ALB) were decreased and the aspartic acid transaminase (AST) and alanine transaminase (ALT) were increased till 5th week. After the cell injection, TP, ALB, AST and ALT were gradually recovered to the same level as the vehicle control group till 9th week. In the 1st week and 4th week after the cell injection, three rats of the each group were sacrificed to get the liver sample for the histological examination and in situ hybridization. In the 5th week after the DMN injection, fiber and collagen in the liver was shown near the portal triad and marginal region. In the 4th week after the cell injection, fibrosis and collagen reduction was considerably observed in the UCB-MSC treated group. To confirm whether UCB-MSC can contribute to reduction of liver fibrosis, we performed in situ hybridization to detect human specific Alu gene. This result showed that human specific gene was detected in the liver parenchymal cells of rats with liver fibrosis after injection of UCB-MSC. In death rate, UCB-MSC and AD-MSC treated rats were shown to decrease, significantly. Taken together, it is suggested that adult human MSCs may have a good therapeutics for the treatment of liver fibrosis.
Zang Hee Lee

Abstract P44

Prostaglandin E2 and HDAC inhibitors induce osteoblast differentiation

Hyunil Ha, Ha-Neui Kim, Jong-Ho Lee, Kyoungsuk Jeong, Han Bok Kwak, Jea Seung Ko, Zang Hee Lee

Department of Cell and Developmental Biology, Seoul National University School of Dentistry, Seoul, Korea

Runx2 is an essential regulator of osteoblast differentiation. Recently, HDAC inhibitors has been reported to increase Runx2 activity and suggested as the therapeutic agents against bone diseases. Here, we screened many kinds of HDAC inhibitor to induce osteoblast differentiation in human mesenchymal stem cells and C2C12 cells. We found that some inhibitors could increase alkaline phosphatase activity in those cells and suppress myogenic differentiation of C2C12 cells.

During the orthodontic tooth movement, the teeth are under inflammatory state. So, proinflammatory cytokines primarily related with bone resorption via osteoclastogenesis, also can affect the opposite side of tooth movement, bone formation. However, There is few studies about the effects of proinflammatory cytokines such as prostaglandin E2 on the PDL inducing differentiation of osteoblasts. In this study, the human PDL cells were treated with prostaglandin E2 and it was evaluated whether the prostaglandin E2 makes the PDL cells induce the differentiation of osteoblast.
Justin Lees

Abstract P45

Optimizing human embryonic stem cell adhesion and single cell survival to improve 3D culturing conditions for the development of β-cell surrogates.

Justin Lees¹, Steven Gao¹, Tristan Croll², Justin Cooper-White², Bernard Tuch¹

¹ The Diabetes Transplant Unit, The Prince of Wales Hospital/The University of New South Wales; ² Tissue Engineering and Microfluidics Group (TE&M), Department of Chemical Engineering, The University of Queensland.

Although ectoderm selection protocols used for the differentiation of embryonic stem cells (ESCs) into β-cell surrogates have achieved some success, alternative directed differentiation strategies are now being considered. It is logical to utilize knowledge of the ontogeny of human β-cells and incorporate that knowledge into the design of differentiation strategies.

We have differentiated human embryonic stem cells (hESCs) in a monolayer to form epithelial cell types that stain for E-cadherin and express Sox-17 and HNF3ß, which are markers of the earliest known β-cell progenitor tissue definitive endoderm. The subsequent stages of β-cell development involve the formation of 3D islet clusters. We have incorporate 3D cell culturing into our β-cell differentiation strategy. 3D scaffolds produced from PLGA were coated with laminin to aid cellular adhesion. hESC derived epithelial cells were seeded onto scaffolds before being transplanted between the liver lobules of mice. In vivo hESCs expressed markers of differentiation including PDX-1 and insulin. The scaffold constructs became vascularised and hESC formed there own endogenous extra-cellular matrix. However, remnant pluripotent cells led to the development of teratomas after extended periods in vivo.

We are now focused on improving our in vitro 3D cell culturing techniques and developing ways of optimizing the seeding of 3D scaffolds with non-tumerogenic hESC derived pancreatic progenitors. We have begun to investigate adhesion and single cell survival of hESCs. The aim is to optimize conditions for the adhesion of single cell suspensions over the entire surface of a 3D scaffold. We have compared the adhesion efficiency and single cell survival of hESCs in various media conditions and in the presence of factors such as extracellular matrix proteins. Our results show that there are significant differences in cell adhesion and survival.
Ji Young Lim

Abstract P46

STELLAR Is Essential for Early Endoderm Differentiation, but not for Maintenance of Self-renewal of Human Embryonic Stem Cells

Ji Young Lim, Jong Joo Lee, Su Youne Han, Yoon Shin Cho and Chul Geun Kim

Department of Life Science, Hanyang University, Seoul 133-791, Korea

Stem cells are unique cell populations having the remarkable potential to develop into many different cell types in the body. To identify genes implicated in the control of the stem cell state as well as the characteristics of each stem cell line, we have analyzed the expression profiles of genes in hESC, hHSC, hMSC using cDNA microarrays, and identified genes that were differentially expressed in specific stem cell populations (Kim et al., 2006). Among genes highly abound in hESCs, we determined here the functional role of STELLAR in self-renewal of hESCs with a lentiviral knockdown strategy. STELLAR (DPPA3, PGC7 in mice) has been reported as the first marker of the specified germ cell lineage, distinguishing the germ cell population from the epiblast. Transduced hESCs were monitored by GFP expression under the fluorescence microscope. The expression level of STELLAR in transduced cells was indeed reduced to about 70% of the wild type as monitored by real-time RT-PCR. STELLAR knockdown cells were maintained their undifferentiated state for more than twenty-five weeks with reduced expression of STELLAR, suggesting that STELLAR is dispensable for the maintenance of self-renewal of hESCs. Potential involvement of STELLAR in hESC differentiation was analyzed by monitoring lineage specific gene expression during EB differentiation. An endoderm marker -fetoprotein was dramatically obstructed in its expression during EB differentiation with no significant deviation of other lineage specific markers. Thus, our data suggest that STELLAR is essential for the fate decision of endoderm differentiation.
Neurotrophin-3 induced early stage of neuronal differentiation by MAPK kinase in neural stem cells

Myung-shin Lim¹, ², Sun-Jung Kim¹, ², Yong-Soon Lee¹, ², Kyung-Sun Kang¹, ²*

¹ Adult Stem Cell Research Center
² Laboratory of Stem Cell and Tumor Biology, Department of Veterinary Public Health, College of Veterinary Medicine and BK21 Program for Veterinary Science, Seoul National University, Seoul, Korea

Neurotrophin-3 (NT-3) is well-known to play an important role in facilitating neuronal survival and differentiation during the development. However, the influence of NT-3 in the early stage in mouse neural stem cells (NSCs) has not been investigated in detail. In this study, we report on three characteristic mechanisms in the early stage about the forms of neurite outgrowth and neuronal differentiation by NT-3 in mouse NSCs. After the NT-3 treatments for 12hr, NSCs were differentiated into more neurons than untreated cells. This indicates that NSCs were expressed in the early stage by the NT-3. It demonstrates that NT-3 stimulation causes NSCs to differentiate into neurons through a phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway and phosphorylated extracellular signal-regulated kinase (ERK) pathway. In addition, the NT-3 treatment induced neurite outgrowth by specific phosphorylation of p38 MAPK which was accompanied with neuronal differentiation. A specific inhibitor of LY294002, PI3K which are upstream to Akt, blocked the phosphorylation of Akt which did not affect the differentiation of NSCs into neuron in response to NT-3 stimulation, whereas the administration of PD98059, an inhibitor of MEK, inhibited the differentiation of NSCs into neurons which was induced by NT-3. Taken together, these results suggest that NT-3 may lead not only to the neural survival through an Akt signaling pathway but also to the neuronal differentiation of NSCs via two distinct downstream signaling pathways in the early stage of NSCs.
Todd Macfarlan

Abstract P48

Analysis of REST and its Corepressors Function in Neurogenesis

Todd Macfarlan¹, Soo-Kyung Lee¹, Yan Zhang², Thomas J. Baiga², Gordon Gill³, Su-Chun Zhang⁴, Joseph P. Noel², and Samuel L. Pfaff¹

¹ Gene Expression Laboratory, The Salk Institute, La Jolla, California 92037
² Jack H. Skirball Center for Chemical Biology and Proteomics, The Salk Institute, La Jolla, California 92037
³ Department of Medicine, University of California San Diego, La Jolla, California 92037
⁴ Departments of Anatomy and Neurology, School of Medicine, and the Waisman Center, University of Wisconsin-Madison, Wisconsin 53705

To ensure that neuronal cell fates are properly segregated during development, transcription factor repressor systems exist to restrict neuronal genes from being expressed too early and in non-neuronal cells.

The RE-1 silencing factor REST has emerged as a critical transcription factor for controlling global level neuron gene expression. REST is a zinc finger protein that binds to a consensus DNA element (called RE1) found near the promoters of ~1000 neuronal genes. In non-neuronal cells, REST recruits a growing list of corepressors to establish a repressed chromatin state. Less is known about the function of REST and its corepressors in stem cells and neuronal progenitors. Since some of the corepressors have enzymatic activities, they may serve as drugable targets. We have therefore begun to analyze the expression and function of REST corepressors in neurogenesis with particular emphasis on the small CTD phosphatase SCP1. We have obtained the crystal structure of SCP1 bound to a CTD peptide and are using this information for structure based inhibitor design. We have also begun to explore the potential of using hES cell differentiation into motor neurons as a tool to study factors that regulate neurogenesis and as a testing ground for pro-neural drugs.
Asif Maroof

Abstract P49

Derivation of Telencephalic, GABAergic Neuronal Subtypes generated from Mouse Embryonic Stem Cells

Asif M Maroof, Lorenz Studer, and Stewart A Anderson

Weill Medical College of Cornell University, New York, NY, USA

In the formation of cortical circuitry, a vast array of neuronal subtypes establish a delicate balance of excitation and inhibition giving rise to the complexity of the nervous system. By providing the major source of inhibitory circuitry in the cerebral cortex, GABAergic neurons are required for the synchronous activity necessary to generate sustained oscillations among neurons within a network that are essential during perception, coordinated movement, learning and memory. Cortical deficits in inhibitory neuronal transmission have been implicated in neuropsychiatric disorders such as epilepsy, autism, and schizophrenia. Generating a limitless supply of GABAergic neurons from embryonic stem (ES) cells would allow for the extensive use of these cells in a variety of disease models in order to evaluate their potential for neuronal repair in the adult CNS. In this study, we directed mouse ES cells toward a ventral telencephalic fate using the SFEB differentiation protocol. Using the morphogens FGF2, FGF8, and SHH, we generated neural progenitors expressing FoxG1, Nkx2.1, and Lhx6, all of which are transcription factors expressed in the medial ganglionic eminence, the major source of GABAergic interneurons. After optimizing the differentiation conditions, the ES-derived neural progenitors were transplanted onto a cortical feeder isolated from neonatal mouse brains. In order to provide enough time for these neuronal progenitors to express markers for specific interneuronal subtypes, we found that the ES-derived progenitors gave rise to several GABAergic interneuronal phenotypes, including GABA, somatostatin, parvalbumin, neuropeptide Y, calbindin, and calretinin. These experiments show that neurochemically-defined neuronal populations can be generated from embryonic stem cells.
Marek Michalak

Abstract P50

Cardiac differentiation of calreticulin-deficient embryonic stem cells

Marek Michalak, Virginie Martin, Pascal Gelebart, Michel Puceat, Marisa Janconi, Michal Opas

Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada

Calreticulin is an ubiquitously expressed calcium binding chaperone of the endoplasmic reticulum (ER). Calreticulin deficiency in mice is lethal in utero due to defects in heart development and function. Calreticulin-deficient embryonic stem (ES) cells can not be efficiently differentiated into cardiomyocytes and show impaired myofibrillogenesis. Cardiac differentiation of calreticulin-deficient cells can be restored by expression of full length calreticulin or calreticulin domain involved in Ca buffering in the ER. This indicates that ER Ca stores may play an important role in cardiac differentiation of ES cells. Molecular analysis of calreticulin-deficient ES-cell revealed that they have impaired ER-dependent Ca homeostasis. This, in turn, affects Ca-dependent transcriptional processes. Immunocytochemistry, Western blot and transcriptional analysis revealed that in calreticulin-deficient cells both MEF2C and NF-AT transcription factors are not efficiently translocated to the nucleus. In this study we show that this is due to a low or no activity of Ca-dependent phosphates calcineurin and to a reduced activity of Erk signaling pathway in calreticulin-deficient ES cells. We concluded that calreticulin and Ca buffering in the ER are essential for normal cardiac differentiation of ES cells.
A genome-wide RNAi screen for asymmetric cell division in *Drosophila*

Jennifer Mummery Widmer, Masakazu Yamazaki, Georg Dietzl, Krystyna Keleman, Doris Chen, Barry J. Dickson, and Juergen A. Knoblich

One mechanism stem cells use to balance self-renewal and differentiation is asymmetric cell division (ACD). In this process, two cells are born. One maintains stem cell identity, self-renewal and proliferation potential, and the other initiates differentiation. During ACD, proteins called cell fate determinants (CFD) are inherited by only one of the two daughter cells. Tight control of unequal CFD segregation between the self-renewing and differentiating daughters is required to fend off uncontrolled proliferation and cancer. *Drosophila* CFD such as Brat and Prospero are suppressors of self-renewal and promoters of terminal differentiation in the non proliferating daughter cell and act by regulating transcription and translation of cell-cycle control genes. If CFD are incorrectly segregated, tumorous growth occurs. Despite the importance of asymmetric protein localization in stem cell biology, its molecular mechanism is not well understood.

To identify novel members of the ACD machinery we carried out an *in vivo* RNAi screen at the genome-wide level, using the *Drosophila* external sensory (ES) organ lineage as a model. ES organs arise from single sensory organ precursor (SOP) cells, which segregate CFD in a series of ACDs. Symmetric SOP divisions result in externally visible cell fate transformations and we used this as a basis for our primary screen. The transgenic fly library we used contains about 20,000 lines with inverted repeats targeting each gene in the *Drosophila* genome. Their expression is controlled by the Gal4-UAS system, allowing tissue-specific gene knock-down. We expressed the hairpins in SOP and their surrounding epithelial cells during development and identified a phenotypic class containing 16 genes known to be involved in ACD and 119 novel genes. We are now carrying out a secondary screen with live imaging of SOP divisions *in vivo*, using a reporter construct for CFD localization to identify candidate genes directly involved in the localization process.
Calreticulin (CRT) is a multifunctional, Ca\(^{2+}\)-storage protein of the endoplasmic reticulum. Its major roles include the regulation of intracellular Ca\(^{2+}\) homeostasis, gene expression, cell adhesion and chaperoning. CRT-null mice die in utero at embryonic day 14.5 due to severe cardiac defects, thus CRT is crucial for cardiogenesis. Interestingly, while the protein is necessary for heart development, it is only a minor component of adult cardiac muscle. Cardiac muscle mass increases with hypertrophy and the walls of the chambers, usually the ventricles, thicken, as mature cardiomyocytes cannot undergo proliferation. Cardiac hypertrophy is initially a compensatory reaction to disturbances such as hypertension, ischemic heart disease, valvular insufficiency, infectious agents or mutations in sarcomeric genes. If the hypertrophic state is prolonged, it leads to arrhythmias, cardiac arrest and sudden death. During cardiac hypertrophy, when the cardiomyocytes make an attempt to achieve proliferative capacity, they re-express many fetal genes. Due to the embryonic lethality of \textit{crt} -/- mice, embryoid bodies were used to investigate the development of cardiomyocytes from embryonic stem cell lines in which \textit{crt} has been ablated vs. their wild type counterparts. Here we show that cardiac proteins are expressed earlier in \textit{crt}-/- cardiomyocytes compared to the wild type cells. Furthermore, the hypertrophic response caused by endothelin-1 or phenylephrine is greater in the \textit{crt}-/- cardiomyocytes. Thus, our working hypothesis is that CRT is playing a protective role during cardiac hypertrophy.
Immunomodulatory properties of canine adipose derived mesenchymal stem cell

Jung Won Kang\textsuperscript{1,3}, Hye Cheong Koo\textsuperscript{1,3}, Young Gun Lee\textsuperscript{2,3}, Yun Hyeok Jung\textsuperscript{2,3}, Young Kyung Park\textsuperscript{1,3}, Kyung-Sun Kang\textsuperscript{2,3} and Yong Ho Park\textsuperscript{1,3}

\textsuperscript{1} Department of Microbiology, \textsuperscript{2} Department of Public Health, \textsuperscript{3} KRF Zoonotic Disease Priority Research Institute, College of Veterinary medicine, Seoul National University.

Like mesenchymal stem cell from bone marrow, adipose tissue (AD-MSC) derived mesenchymal stem cells can differentiate into several lineages and have immunomodulatory properties. In this study, we isolated canine AD-MSCs and investigated their phenotype, cytokine expression, and immunomodulatory effects on lymphocyte proliferation for characterization of newly established canine MSCs. We determined the surface marker expression by flow cytometric analysis, the cytokine mRNA expression by reverse-transcriptase polymerase chain reaction (RT-PCR), lymphocyte proliferation activity by mixed lymphocyte reaction, and cytokine production by ELISA. AD-MSCs revealed to be positive for the endoglin receptor (CD44, CD90) and hematopoietic stem cell marker CD34 and negative for the lipopolysaccharide receptor CD14, the leukocyte common antigen CD45, and other hematopoietic markers (CD3, CD4, CD8, sIgM, CD172a, CD11c, HLA-DR). They expressed the mRNA of cytokine (TGF-beta, IL-6), chemokine (IL-8, MCP-1, RANTES), growth factor (VEGF), protease inhibitor (TIMP-1, TIMP-2), COX-2 but no mRNA of IL-10, TNF-alpha, MMP-9 and etc. Furthermore, the proliferation of lymphocyte to mitogen was suppressed not only by culture supernatant of AD-MSCs and co-culture with irradiated AD-MSCs but also by AD-MSCs separated by transwell. Moreover, the TNF-alpha production was significantly decreased by AD-MSCs. In conclusion, these findings support that canine AD-MSCs have immunomodulatory properties with their potential therapeutic use in immune-mediated disorder. Thus, further study is needed to identify the detailed mechanism implicated in immunomodulatory effects of AD-MSCs.
Epigenetic Reprogramming of Adult Human Germ Cells

Francis Pau, Francisco Silva, Fari Izadyar, Natalia Slepko, Rafael Gonzalez, Joel Marh, Thomas Ramos, Kyle Howerton, Jane Pham, Jason Pacchiarotti, Michael Pascual, Chad Maki, Carl Javier, Susanne Csontos, Jadelind Wong, Sandra Anorve, Mauricio Umana, Chauncey Sayre

PrimeCell Therapeutics LLC, 213 Technology Drive, Irvine, CA 92618, USA

Our laboratory has shown that adult mouse germ cells subjected to culture-induced reprogramming can regain their pluripotentiality, including up-regulation of Oct-4 expression, exhibition of pluripotent markers such as Nanog, SSEA-1 and alkaline phosphatase, differentiation into phenotypes derived from all three germ layers and formation of chimeric cell populations in the brain, heart and testis after blastocyst injection and implantation in host mice. We report here that adult human germ cells subjected to epigenetic reprogramming also acquired pluripotent stem cell characteristics. Adult human germ cells were isolated by enzymatic dissociation and enriched by differential adhesion, by separation in Percoll gradients, by fractionation in bovine serum albumin gradients, or by cell sorting. They were subjected to several culture conditions containing various growth factors in different concentrations, various amount of fetal bovine serum (FBS), or different substrates for cell adhesion. We found that enriched adult human germ cells continued to grow in a serum-free medium containing reprogramming growth factors (PM-10™) for at least 2 months. Cells in these populations exhibited Oct-4 up-regulation, expressed Nanog and other pluripotent markers, and spontaneously differentiated into cardiomyocytes, neural cells and adipocytes. The addition of sonic hedgehog and FGF-8 into the PM-10™ medium initiated neural differentiation. Addition of 5'-aza-3'-cytidine promoted cardiomyocyte differentiation. Addition of PDGF-BB enhanced cell growth, whereas addition of BMP-4, BMP-7 or FBS (1% to 15%) induced differentiation. Co-culture with STO cells, mouse embryonic fibroblasts, hematopoietic stem cells and human liver cells all promoted differentiation. Cells sorted for SSEA-4 and/or Thy-1 grew into small colonies in the PM-10™ medium when co-cultured with mouse or human feeder cells. Fibronectin promoted cell adhesion comparing to gelatin. We conclude that the PM-10™ is an effective medium for adult human germ cell growth and reprogramming. The efficiency of the PM-10™ medium can be improved and optimized by modifications of the culture environment.
Reliable and abundant cell sources are needed for the regeneration of bone and cartilaginous tissues associated with a variety of musculoskeletal injuries and disorders. Adult mesenchymal stem cells (MSCs) have been widely studied; however, these cells are lineage restricted and expansion is limited.

Amniotic fluid stem cells (AFSCs) have recently been isolated from human and murine amniotic fluid after routine amniocentesis. Preliminary reports suggest that the AFSCs have the capacity to differentiate into all three embryonic lineages, much like embryonic stem cells, but without the use of murine feeder layers. However, unlike embryonic stem cells, teratoma formation has not been observed thus far with the implantation of AFSCs in vivo.

These cells are therefore an exciting new potential source for cell therapy and tissue engineering. The purpose of this study was to begin investigating the potential of AFSCs to differentiate into osteoblasts as a step towards engineering 3D tissues using this novel cell source.

We hypothesized that AFSCs would undergo extensive osteogenic differentiation, but, due to their more primitive state, would demonstrate a delayed mineralization response as compared to MSCs. Initial studies show increased mineralization by the MSCs when compared to the AFSCs at early time points. As the culture continued, the mineral produced by the AFSCs was comparable to the MSCs. Additionally, the rate of mineral production between 5 and 10 weeks was significantly higher in the AFSCs than the MSCs.

3D culture of AFSCs show extensive mineralization of PCL scaffolds after 12 weeks in osteogenic differentiation media. The robust mineralization of the AFSCs will allow 3D tissue constructs to be produced for future in vivo evaluation in a preclinical model.

This study shows the remarkable potential of amniotic fluid stem cells for the treatment of bone disorders.
Laura Perin

Abstract P56

In vitro and in vivo application of Amniotic Fluid Stem Cells (AFSC) for Renal Tissue Regeneration

Laura Perin, Stefano Giuliani, Daniel jin, Sargis Sedrakyan, Gianni Carraro, Steve Kim, Casey Brewer, Anthony Atala, Rofer De Filippo

Childrens Hospital Los Angeles; Saban Research Institute, 4661 Sunset BLVD, 90027 Los Angeles, USA

Aim of study: A growing shortage of donor organs has heightened interest in developing novel strategies for tissue regeneration, including the possible role of stem cells, as an alternative to heterologous organ replacement in patients with renal failure. This work seeks to explore a unique population of human and mouse amniotic fluid derived stem cells (hAFSC, mAFSC) and determine their capacity to differentiate into renal cells by direct injection into embryonic kidneys using inductive pathways of renal embryogenesis and development in an in vitro system. Furthermore, we proceed with the in vivo application of AFSC to test their ability to survive, replicate and integrate in the kidneys of a nude mouse model.

Methods: A pluripotential stem cell population exists in amniotic fluid. These cells express stem cell markers (Oct-4, SSEA-4), are clonogenic, and are able to differentiate into all three germ layer derivatives in vitro. For the in vitro experiments embryonic kidneys were dissected from mouse embryos 13.5 days of gestation and gfp or lac-z labeled hAFSC are directly injected into the organs. The kidneys were then cultured and fixed at specific time points, and were analyzed by histology, RT-PCR and live imaging microscopy. For the in vivo experiments a luc-mAFSC population was injected (tail vein injections and open surgery) into nude mice. The animals were monitored using bioluminescent detection at specific time points and then were scarified for histological analysis to determine the ability of AFSC to survive and replicate and differentiate in vivo.

Main results: Histological analysis of serial sections showed that the AFSC were able to contribute to kidney structures. Once injected into embryonic kidneys, the AFSC were able to survive, replicate, and follow the natural branching pattern of the developing kidney integrating into kidney primordia. RT-PCR demonstrated that these cells complete an initial step essential for commitment to a renal fate during organ culture. Preliminary data from in vivo experiments demonstrated that AFSC could survive after tail vein injection (luciferase detection). Histologic analysis of the in vivo specimens showed that mAFSC could survive, integrate and differentiate into kidney structures, expressing tubular and glomerular markers.

Conclusion: This preliminary data suggest that AFSC have the capacity to survive, replicate, and integrate once injected into embryonic kidneys. The preliminary in vivo data suggest that AFSC can survive and integrate once injected into an animal model. In addition the cells are able to differentiate into nephrons structures. AFSC could, therefore, represent a limitless source of ethically neutral, unmodified stem cells that may prove useful as a novel alternative for whole organ regeneration or cell therapeutic techniques in the future.
Sharla Phipps

Abstract P57

Differential expression of epigenetic modulators during human embryonic stem cell differentiation

Sharla M. O. Phipps, Hsu-Kun Wang, Troy E. Mott, Joel B. Berletch, Lucy G. Andrews, Trygve O. Tollefsbol

1300 University Blvd, CH 175, Birmingham, AL 35294-1179

Although the progression of aging and the diseases associated with it are extensively studied, little is known about the initiation of the aging process. Links between DNA damage, telomeric attrition, and cellular senescence have been established in vitro and point to a molecular clock that governs the processes of aging. A major question in telomerase regulation centers on this holoenzyme being down-regulated early in embryonic differentiation thereby leading to telomeric attrition and aging. The mechanisms underlying the coupled inhibition of telomerase activity and programmed cellular differentiation remain elusive, but epigenetic studies in differentiating human embryonic stem (hES) cells could potentially give clues about how and when DNA methylation and histone deacetylation work together to inactivate hTERT at the onset of the aging process. Using immunohistochemistry on cultured hES cells, we have shown a stronger correlation between hES differentiation and expression of the epigenetic regulators DNMT 3a and 3b than between genetic modulators of differentiation such as Mad1 and p53. Such studies could have significant importance not only pertaining to the early events involved in aging and telomere shortening, but also in regard to potential intervention for life extension through the use of compounds that modulate epigenetic processes.
In this study, we show evidence that lymphangiogenesis and development of peripheral nervous system (PNS) are aligned processes. Vascular endothelial growth factor C (VEGF-C) is essential for lymphangiogenesis by promoting migration of lymphatic endothelial cells (Karkkainen et al. 2004). Vegfc-deficient mouse embryos have also a loss of oligodendrocyte precursors in optic nerve (Le Bras et al. 2006). However, the effect of VEGF-C on developing PNS has not been studied in detail. In this paper we studied a role of VEGF-C in PNS by using Vegfc lacZ and Wnt1-cre/R26R embryos at developmental stages E10-13, sympathetic ganglia chain derived progenitors (SNPCs) and neural crest derived neuroblastoma cells (N2A). We show that VEGF-C co-localizes temporally and spatially in areas with developing sympathetic ganglia chain (SGC) and Vegf-C deficiency leads to decreased number of neurons in SGC. In vitro VEGF-C increases cell proliferation and survival of SNPCs and N2A cells. However, migration, differentiation, or neuron outgrowth of SNPCs is not affected. C-RET receptor tyrosine kinase is implementary in the VEGF-C responsiveness of the sympathetic ganglia chain. We propose that VEGF-C is involved in aggregation and growth of sympathetic ganglia by promoting the proliferation and survival of the progenitors.

References:
The role of a stem cell specific transcription factor in pluripotency and self-renewal

Janine N. Post, Wiebe Kruijer

Molecular Cell Biology, Faculty of Science and Technology, Universtity of Twente, P.O.Box217, 7500AE, Enschede, The Netherlands

The molecular mechanisms by which stem cells control self-renewal and differentiation are still poorly understood. However, evidence points to an important role for epigenetic reprogramming and chromatin remodeling in these processes. Chromatin remodeling is regulated by intra-cellular as well as extra-cellular processes and is a key factor in the regulation of gene transcription. This control of gene transcription may well be the most important factor in stem cell renewal and determination of stem cell fate. We study the role of signaling from i) soluble factors in the niche, ii) cell-cell contact and iii) cell-matrix contact, as well as the cross-talk between these pathways in the maintenance of pluripotency and differentiation.

Here we report the influence of a stem cell specific transcriptional co-factor on chromatin remodeling and expression of TGF / BMP specific target genes. Our experiments show that the co-factor is able to bind directly to DNA. Moreover, ChIP assays show that the binding of this transcriptional co-factor to the promoters of target genes of the TGF signaling pathway is influenced by TGF-R activation. In addition, this transcriptional co-factor is able to bind HDAC1 as well as ATF2 (HAT), indicating that its binding can directly influence chromatin structure and transcription of these TGF / BMP target genes. The implication of these findings for target gene regulation and maintenance of self-renewal in both adult and embryonic stem cells will be discussed.
Sonic hedgehog (Shh) and Wnt-7a are secreted morphogens involved in the embryonic development of the vertebrate forebrain as well as in neurogenesis in the adult brain. GFP-labeled P0 mouse neural stem cells expressing either Shh (Shh/GFP) or Wnt-7a (Wnt-7a/GFP) were used to study their effect on neural stem cell proliferation and differentiation in vitro. Electrophysiological characterisation using the patch-clamp technique and immunohistochemical analysis were carried out 8 days after the induction of differentiation by retinoic acid; wild-type cells (WT/GFP) were used as a control. In WT/GFP cells three distinct cell populations were identified. Large flat cells with a cell-body diameter of 40µm formed an underlying layer (19%), expressed GFAP, and displayed passive, time- and voltage-independent K+ currents, with an average membrane potential (Vm) of -87 mV and input resistance (IR) of 61MOhms. The second population of cells (16%) with a triangular cell-body (diameter 25 µm) expressed GFAP or NG2 and predominantly displayed passive, time- and voltage-independent K+ currents together with an inwardly rectifying current activated by hyperpolarisation. Their mean Vm and IR were -90 mV and 72 MOhms, respectively. The third group of cells (65%) with a cell-body diameter of 15µm (termed neuron-like cells) were MAP-2, DCX or β-III tubulin positive with a mean Vm of -83 mV and IR of 357 MOhms and mostly displayed voltage-dependent K+ currents together with an inwardly rectifying current activated by hyperpolarisation. Shh expression led to increased numbers of both flat and triangular cells, but their passive membrane properties were not significantly different from those in control cells. The number of neuron-like cells decreased by 25%, and moreover, I Na currents were not detected. In Wnt-7a/GFP cells the number of large flat cells was decreased by 16% and the number of triangular cells increased by 23%, while the number of neuron-like cells was not significantly different when compared to controls. All three cell populations showed increased IR and decreased Vm; further, in neuron-like cells the I Na amplitude was significantly increased. Based on electrophysiological data we can conclude that Wnt-7a/GFP cells showed marked differences compared to Shh/GFP and WT/GFP cells, thus these morphogens might play an important role in postnatal neural stem cell differentiation.

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The role of FGF2 signaling pathway in the stemness of human embryonic stem cells

Jeung-Yon Rho\textsuperscript{1}, Jung-Jae Shim\textsuperscript{1}, Jee-Soo Han\textsuperscript{2}, Janghwan Kim\textsuperscript{2} and Yong-Mahn Han\textsuperscript{1}\textsuperscript{*}

\textsuperscript{1}Department of Biological Sciences, Korea Advanced Institute of Science and Technology (KAIST), Daejeon 305-701, Korea; \textsuperscript{2}Center for Development and Differentiation, Korea Research Institute of Bioscience and Biotechnology (KRIIBB), Daejeon 305-806, Korea

The undifferentiated human embryonic stem cells (hESCs) are maintained by culturing in the culture medium supplemented with basic FGF (FGF2). This study was conducted to understand the function of FGF2 signaling pathway in the stemness of hESCs. hESCs (SNU-hES3) were cultured in the ES culture medium on mitomycin C-treated STO feeder cells. ES culture medium was DMEM/F12 medium containing 20% knockout serum replacement. After treatment of hESCs with an inhibitor of FGFR tyrosine kinase SU5402 (10 μM) for 5 days, transcriptional expression of \textit{Oct3/4} and \textit{Nanog} genes was downregulated. Interestingly, expression of FGFR3 increased remarkably in SU5402-treated hESCs as compared to the non-treated hESCs, but no difference was detected in the expression of FGFR1. Expression levels of c-myc and Cyclin D1 genes were lower in SU5402-treated hESCs than in normal hESCs. In the next experiment, we investigated the expression of downstream genes related to PI3K and MAPK signaling pathways which are affected by FGF2 signaling. PI3K regulatory subunit was upregulated in SU5402-treated hESCs, but there was no difference in the expression of PI3K catalytic subunit. Transcriptional expression levels of many factors including Gab1, AKT, mTOR and p70S6K except E-Ras, which are related to PI3K signaling pathway, were not affected by SU5402 treatment. Among intermediate molecules related to the MAPK signaling pathway, phosphorylated p-Erk and p-Elk were specifically enhanced in SU5402-treated hESCs. In addition, some mesoderm lineage-marker genes such as Brachyury and GATA2 were highly transcribed in SU5402-treated hESCs. Consequently, our results indicate that FGF2 signaling regulates high expression of c-myc and Cyclin D1 by activating the PI3K and MAPK signaling pathways, thereby maintaining the stemness of human embryonic stem cells.

Key words: basic fibroblast growth factor/ phosphatidylinositol 3- kinase/ mitogen-activated protein kinase/ c-myc/ cyclin D/ human embryonic stem cells
Sarah Robins

Abstract P62

Analysis of Stem / Progenitor Cells in the Adult Mouse Hypothalamus

Robins S, Ohyama K, Ellis P, Placzek M.
Placzek Lab, D223, Alfred Denny Building, University of Sheffield, Western Bank, Sheffield, S10 2TN

Neurogenesis continuing throughout life has been well-documented in the dentate gyrus and subventricular zone, but new evidence is emerging that stem cells may also be present in other regions of the adult brain. It has been proposed that one such population of neural stem cells may reside in the hypothalamus, more specifically in the ependymal layer of the third ventricle (Xu, Tamamaki et al. 2005). To begin to extend such studies we have used immunohistochemical analyses to investigate the expression of stem/progenitor cell markers in the adult mouse hypothalamus, examining expression of Sox1-3, musashi, nestin and BLBP. In addition, we have isolated proliferative cells from the hypothalamus of adult mice, and cultured them as neurospheres. By examining putative stem cell marker expression and by long-term passaging of these neurospheres we aim to determine whether they are self-renewing stem cells or restricted progenitors. We are studying the properties of these neurospheres, to determine if they can differentiate to produce a mix of cell types. Currently, we have demonstrated the presence of neurons of several different subtypes, glia and oligodendrocytes. In addition, we are asking how the neurospheres differentiate in the presence of a range of hormones, growth factors and signalling molecules, with preliminary studies suggesting that some of these influence cell fate and migration. To complement this line of work, we are conducting in vivo experiments involving neurosphere transplantation into chick embryos to assess their ability to integrate into different neural and non-neural areas. Both experimental approaches should help ascertain whether these cells are intrinsically hypothalamic in nature, or whether they are conferred hypothalamic character by spatial cues.

Vladimir Ruzhynsky

Abstract P63

Crosstalk between cell cycle regulator E2F4 and morphogene Shh

Vladimir Ruzhynsky, Kelly McClellan, Jacqueline Vanderluit, Marosh Furimsky, David S. Park, Valerie Wallace and Ruth S. Slack

Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa Health Research Institute - Neuroscience Program, 451 Smyth Rd, Ottawa, Ontario, Canada K1H 8M5.
Molecular Medicine Program and Vision Program, Ottawa Health Research Institute, 501 Smyth Road, Ottawa ON K1H 8L6; Department of Biochemistry, Microbiology and Immunology, University of Ottawa, 451 Smyth Road, Ottawa, Ontario, Canada K1H 8M5.

The Retinoblastoma family proteins control cell cycle progression by regulating the activity of E2F transcription factors. E2F4 plays an important role in the control of terminal mitosis and differentiation in a number of developing systems. The goal of these studies is to examine the role of E2F4 during the mammalian brain development and neural stem cell regulation. E2F4 null embryos display a dramatic reduction of ventral telencephalic structures and decreased self renewal capacity of neural precursors. Examination of the Sonic Hedgehog (Shh) pathway revealed a defect in Shh signaling in the ventral telencephalon. To determine whether there is a genetic interaction between E2F4 and Shh we generated compound mice heterozygous for both E2F4 and Shh. While both single heterozygotes developed normally, the compound heterozygote for E2F4/Shh exhibited a dramatic loss of ventral telencephalic structure similar to E2F4 null mice. Consistent with a defect in Shh signaling, loss of one allele of the Shh target, Gli3, rescued the ventral defect found in E2F4 null mutants. Addition of a Shh agonist to neural precursor cells could restore self renewal capacity. Similarly, Shh agonist could restore the expression of the ventral marker Nkx2.1 in ex vivo cortical explant culture studies. These results demonstrate a crucial function for E2F4 in ventral telencephalic patterning and reveal a novel signaling interaction between the cell cycle regulatory protein E2F4 and the morphogene Shh.
Antonio Salgado

Abstract P64

Interactions Between Human Umbilical Cord Perivascular Cells (HUCPVCs) and Glial Cells: the role of paracrine factors released into conditioned medium

A.J. Salgado, A.R. Mesquita, N.M. Neves, R.L. Reis, N. Sousa

Neuroscience Group, Life and Health Science Research Institute (ICVS), University of Minho, Braga, Portugal & 3Bs Research Group, University of Minho, Braga, Portugal

In the last years Umbilical Cord Stem/Progenitor Cells have been put forward as possible therapeutic tools for the treatment of severe conditions associated to the central nervous system. Several reports in the literature have shown that these cells were able to integrate within the CNS upon injury and to improve the condition of animals suffering from neurodegenerative diseases, a fact that was attributed to the local increase of neuronal populations. However, and quite surprisingly, the effect of these cells on the local glial cell populations has not been studied. Therefore the main objective of the present work was to understand how a population of umbilical cord progenitor cells, known as Human Umbilical Cord Perivascular Cells (HUCPVCs), interact with a mixed glial cell population, namely at the cell viability, proliferation and differentiation levels. HUCPVCs were isolated from the primitive connective tissue present in umbilical cord known as Wharton Jelly, while glial cells were isolated from the cortices of P5 newborns. Conditioned medium (CM) from both cell populations was collected and frozen on days 3 and 7. HUCPVCs and glial cells where then exposed to opposite CMs for 1 week after which cell viability and proliferation was assessed by the MTS test. Immunocytochemistry against GFAP (astrocytes), O4 (oligodendrocytes) and cd11b (microglia) was also performed to analyse the specific effects of HUCPVC-conditioned medium on each of the cell populations present in the mixed cultures. Cell viability experiments revealed that HUCPVCs incubated with glial-CM and glial cells incubated HUCPVC-CM disclosed levels of metabolic activity, and hence of cell viability, very similar to those found for the control condition. However, immunocytochemistry studies showed that HUCPVC-CM triggered proliferation on all glial sub-populations; importantly, this effect was particularly evident for microglia cells. These results demonstrate the sensitivity of other CNS cells besides neurons, and reveal distinct levels of response within different glia cell populations. Future work will focus on the identification of the growth factors underpinning these processes and consequent crosstalk mechanisms related to the phenomena presented herein, which may pave the way for the development of strategies to control glial cell activity, directing them into relevant tissue regeneration.
Hannu Sariola

Abstract P65

Regulation of spermatogonial stem cell fate by GDNF

Hannu Sariola

Institute of Biomedicine, University of Helsinki, P.O.Box 63, FIN-00014 University of Helsinki, Finland

Millions of sperm cells are produced every day during man’s fertile life. Self-renewal and
differentiation of spermatogonial stem cells (SSCs) must thus be in strict balance. Using
transgenic loss- and gain-of-function mouse models we have shown that Glial-Cell-Line-
Derived Neurotrophic Factor (GDNF) regulates the cell fate decision of SSCs. At low dosage,
the SSCs differentiate in excess and get depleted. At high dosage, the SSCs only self-renew,
form clusters in seminiferous tubules, and are unable to differentiate. The clusters undergo
extensive apoptosis at around 10 weeks of age and the testes become atrophic.

Germ line malignancies start appearing in the testes of GDNF overexpressing mice at one
year of age and finally approximately 90 % of the mice develop testicular cancer. All of them
mimic human seminomas by morphology and marker gene profile, and their karyotype is
triploid like in human seminomas.

Several long-term culture methods for SSCs have now been developed. They are based on
various combinations of media, hormones and growth factors, as well as feeder cells or
laminin coating. With the exception of one published method, all recipes include GDNF.

Now, when long-term culture of SSCs is finally possible, the SSCs can be used for gene
targeting, and the first results have indeed been reported. If similar culture conditions will
maintain human SSCs, cultured SSCs might also provide a possibility to restore fertility of
cancer patients. However, there are controversial results about the multipotency of SSCs
after long-term culture. When SSCs cultured with GDNF have been injected to testes, they
restore sperm production. When SSCs have been cultured without GDNF, they behave like
ES cells and form teratomas. We are currently working on the differences between the SSCs
cultured with or without GDNF, and trying to identify the GDNF-dependent pathways that are
essential to maintain the SSC identity in vitro.
Deciphering the molecular basis of cancer is critical for developing novel diagnostic and therapeutic strategies. To better understand the early molecular events involving osteogenic sarcoma (OGS), we have initiated a program to identify potential tumor suppressor genes. Expression profiling of total RNA from ten normal bone cell lines and eleven OGS-derived cell lines by microarray showed 135-fold lower expression of FRZB/sFRP3 mRNA in OGS cells compared to bone cells; this down-regulation of Frzb/sFRP3 mRNA expression was found to be serum-independent. Subsequently, fourteen OGS-biopsy specimens showed nine-fold down-regulation of Frzb/sFRP3 mRNA expression compared to expression in eight normal bone specimens as determined by microarray. Quantitation by RT-PCR indicated ~70% and ~90% loss of Frzb/sFRP3 mRNA expression in OGS biopsy specimens and OGS-derived cell lines respectively. Hybridization experiments of a cDNA microarray containing paired normal and tumor specimens from nineteen different organs did not show any significant difference in the level of Frzb/sFRP3 mRNA expression between the normal and the corresponding tumor tissues. Exogenous expression of FRZB/sFRP3 mRNA in two OGS-derived cell lines lacking endogenous expression of the mRNA produced abundant mRNA from the exogenous gene, eliminating degradation as a possibility for very low level of FRZB/sFRP3 mRNA in OGS specimens. Results from PCR-based experiments suggest that the FRZB/sFRP3 gene is not deleted in OGS cell lines, however, karyotyping shows gross abnormalities involving chromosome 2 (location of the FRZB gene) in five of twelve OGS-derived cell lines. Together, these data suggest a tumor-suppressive potential for FRZB/sFRP3 in OGS. We further speculate that loss of FRZB expression is an early event in the cancer stem cell important for developing OGS.
MIAMI Stromal Stem Cells Self-Renewal and Differentiation Potentials During Aging


University of Miami Miller School of Medicine and GRECC, Veterans Affairs Medical Center, Miami, FL 33125, U.S.A.; Centre Hospitalier Universitaire d’Angers, and INSERM ERT-M 0104, Angers, France

Human marrow-isolated adult multilineage inducible (MIAMI) are normal stromal cells and have been isolated from males and females 3- to 72-years-old. The developmentally primitive status of these cells is characterized by the sustained expression of embryonic stem cell markers Oct4, Sox2, Nanog, Rex1, SSEA-4, Bmi-1 and hTeRT among other molecules, like Notch1, NTRK-3 and MAP1b above 30 population doublings (PD). Although the frequency of MIAMI cells, among all marrow nucleated cells, decreases from 0.01% at age 3 to 0.0018% at age 45; their numbers appear to remain constant after this age. While the expression of a few genes involved in mitosis and cell cycle regulation appeared decreased and the expression of genes encoding chemokines involved in inflammation appeared increased with aging, the level of expression of the markers characteristic of MIAMI cells (Oct-4, Sox-2, nanog, Rex-1, cMet, NTRK-3, SSEA-4, CD29, CD63, CD81, CD122, and CD164) remained constant independently of age and gender. These data suggest that some cell intrinsic factors may limit the capacity of MIAMI cells to self-renew, proliferate, or progress toward specific differentiation programs. In long-term in vitro expansion experiments of MIAMI cells, above 50 PD, aging increased the PD time by about 20-30%. In contrast, in vitro differentiation of MIAMI cells towards osteoblastic cells (responsiveness and timing) was unaffected. This suggests that the intrinsic factors have a minimal or undetectable effect on the capacity of MIAMI cells to reach Hayflick’s limit or progress toward the osteoblastic differentiation program respectively.

Stimulation with parathyroid hormone can increase either proliferation or osteoblastic differentiation depending on the culture conditions. For stimulation of osteoblastic differentiation, functional gap-junctional communication mediated by connexin43 is required. Inhibition of gap-junctional communication not only blocked the osteoblastic differentiation program but also stimulated the adipocytic differentiation program. This data suggest that cell extrinsic factors, such as those that may interfere with hormonal signaling or gap-junctional communication, could have a greater impact on the self-renewal or differentiation capacity of marrow progenitor cells than cell intrinsic factors during aging.

We have also examined the differentiation potential of MIAMI cells to other lineages. Initial analysis of a limited number of samples indicates that MIAMI cells from young (under 15-years-old) and older (over 45-years-old) maintain their capacity to differentiate to other mesodermal (chondrocytic), endodermal (hepatocytic) and ectodermal (neuronal) lineages. It is interesting to highlight the potential of MIAMI cells to differentiate towards a neuronal phenotype in a sequential manner similar to that observed during neuronal development. During the in vitro neuronal differentiation process, which is dependent on NT-3, the expression of SSEA-4 synthase, Sox2, Notch and MAP1b by MIAMI cells was diminished while that of Neurogenin 2, beta-3 tubulin and neurofilament M appeared. Pre-treatment of MIAMI cells with EGF and FGF-2, two mitogens for neural stem cells, seemed to accelerate this differentiation process. Moreover, with a modified dopaminergic induction protocol MIAMI cells acquired an immature dopaminergic phenotype; they expressed Nurr1 and tyrosine hydroxylase.

This data suggest that MIAMI cells may be useful for the treatment of degenerative diseases related to aging, including Parkinson’s disease. In this context we have conceived a new tool for cell therapy, the pharmacologically active microcarriers, which may transport the grafted cells and induce and/or maintain the differentiation of stem cells after transplantation. The utility of these cells may be particularly relevant to those cases in which decreased or lack of tissue repair is due to a diminished stem cell pool. So the isolation, ex-vivo expansion, and reimplantation of autologous primitive MIAMI stem cells could be a means of repopulating this...
stem cell pool, which decreased during aging, and contribute in this way to recover the tissue repair capacity in older individuals.
Abstract P68

Improvement of 3D Culture Systems for Large Scale Production of Undifferentiated Rat and Human Adult Stem Cells

M Serra¹, S Leite¹, MJT Carrondo¹-², PM Alves¹

¹IBET/ITQB, Apartado 12, 27804-901 Oeiras, Portugal
²FCT/UNL, 2825 Monte da Caparica, Portugal

Adult stem cells are undifferentiated cells found within fully developed tissues or organs of an adult individual [1]. Until recently, these cells have been considered to bear less self-renewal ability and differentiation potency compared to embryonic stem (ES) cells. However, recent findings brought new promises for stem cell research: the appearance of a new lineage class of uncommitted pluripotent adult stem cells with a remarkable self-renewal ability and differentiation potency: the PSLCs (Pancreatic Stellate-Like Cells) [2]. This suggests that adult stem cells equivalent to embryonic stem cells might exist.

To realize the full therapeutic potential of stem cells, in vitro methods for maintaining the unspecialized state of cells, promoting the proliferation of stem cells, and directing the differentiation of stem cells to the needed specialized cells are a challenge. The main goal of this study was the development of expandable culture systems to produce large numbers of highly pure populations of undifferentiated rat and human PSLCs. To achieve this, different strategies were tested:

i) The first strategy was focused on the use of microcarriers to support adult stem cell expansion without differentiation. Optimization of culturing processes was carried out taking into account the following critical parameters: microcarrier concentrations, inoculum size, agitation rate, and operational mode (batch, fed-batch, continuous/perfusion).

ii) During the second strategy, the expansion of undifferentiated PSLCs was evaluated in the form of small 3D cell aggregates. Herein, the size of 3D cell aggregates was strongly monitored and controlled in order to avoid detrimental diffusion gradients and/or spontaneous differentiation.

In this study, spinner vessels (125ml) were used for all 3D culture strategies experiments. Cell growth and viability was monitored daily by DNA content and following the enzyme lactate dehydrogenase activity, respectively. Metabolic performance of cells was assessed from glucose uptake rates and lactate to glucose ratio. Immunostaining tools and telomerase activity assay were performed to assess the efficient maintenance of the pluripotent and undifferentiated state of both rat and human PSLC.

First results show that expansion of undifferentiated adult pancreatic stem cells is possible using Cytodex-1 microcarrier (from GE Healthcare) as a support. Culture of small 3D cell aggregates is under development with preliminary results indicating that these adult stem cells can be cultured in suspension conditions and proliferate as small spherical aggregates. Overall, both strategies are promising tools for scaling-up adult pluripotent stem cells growth in stirred bioreactors by allowing high cell density per unit volume, as well as an efficient control and monitoring of several environmental parameters and easy cell sample harvesting.

Embryonal carcinoma (EC) stem cells derived from germ cell tumours are valuable tools for the study of embryogenesis and closely resemble embryonic stem (ES) cells. The human pluripotent NT2 cell line, derived from a teratocarcinoma, has been used extensively to study neuronal differentiation [1]. These cells can be induced to terminally differentiate into postmitotic neurons (NT2-N) when treated with retinoic acid [2]. Although techniques have been developed to produce and purify EC-derived neurons from other contaminating cell types, these methods are laborious, yield relatively few neurons per culture, and are thus time consuming.

This study reports a novel culture strategy for NT2 cell proliferation and neuronal differentiation in order to control and enhance the efficiency of neuronal production from human EC stem cells. NT2 cells were cultured as 3D cell aggregates (“neurospheres”) in the presence of retinoic acid, using stirred small scale bioreactors where temperature, pH and pO\textsubscript{2} can be monitored and controlled. Cell growth, viability and aggregate size were monitored daily. Also, cryosection immunostaining was performed in order to characterize the NT2 neurospheres during the culture period. Metabolic performance of cells was assessed from glucose uptake rates, lactate to glucose ratio and oxygen consumption. Finally, NT2 neurospheres were collected at different culture times and plated on glass coverslips precoated with poly-D-lysine and Matrigel. After treatment with mitotic inhibitors the adherent cultures were characterized by immunofluorescence microscopy, NT2-N neurons were stained with antibodies anti-neurofilament light subunit and anti-β tubulin III. Non-neuronal cells were stained with anti-nestin antibody.

Such results showed that these novel culture strategies allow a faster and more efficient production of NT2-N neurons. Also, they are promising tools for proliferation and enhanced neuronal differentiation by assuring fully controlled culture conditions (namely, aggregate diameter, pH, pO\textsubscript{2} and temperature). Data will be presented to substantiate a robust system to culture stem cells for regenerative medicine and cell therapy.

Danielle Shing

Abstract P70

The PR domain-negative form of PRDM16 is leukaemogenic

Danielle C. Shing1, Maurizio Trubia2, Francesco Marchesi3, Enrico Radaelli3, Elena Belloni2, Cinzia Tapinassi2, Cristina Mecucci4, Idoya Lahortiga5, Antonio Romano6, Valentina dall'Olio2 and Pier Giuseppe Pelicci1

1 Department of Experimental Oncology, European Institute of Oncology, Milan, Italy; 2 IFOM-FIRC Institute of Molecular Oncology, Milan, Italy; 3 Department of Veterinary Pathology, Hygiene and Public Health, School of Veterinary Medicine, University of Milan, Milan, Italy; 4 Department of Clinical and Experimental Medicine, Section of Clinical Haematology and Immunology, University of Perugia, Perugia, Italy; 5 Department of Genetics, University of Navarra, Pamplona, Spain; 6 Clinical Division of Haematology and BMT Unit, Antonio Perrino Hospital, Brindisi, Italy.

Acute myeloid leukaemia (AML) is initiated and maintained by a population of leukaemic stem cells that are either haematopoietic stem cells that have accumulated oncogenic mutations or committed progenitor cells that have acquired the ability to self renew. The fusion genes or oncogenes that initiate the leukaemogenic process are frequently able to block myeloid differentiation and confer properties of self renewal on the target cell, leading to the accumulation of abnormally differentiated, immature myeloid cells.

We have identified aberrant expression of PRDM16 to be a frequent and recurrent event in AML, occurring in patients with translocations of 1p36 and in a subset of AML with normal karyotype. Two isoforms, PRDM16 and sPRDM16, can be expressed from the PRDM16 gene, differing in the presence and absence of the PR domain. To understand how aberrant expression of PRDM16 contributes to leukaemia, we have determined the biological properties of both isoforms. In vitro, expression of sPRDM16 in murine bone marrow progenitor cells blocks myeloid differentiation, increases self renewal upon serial replating and increases the stem cell pool in a long term culture initiating cell assay. In contrast, PRDM16 behaves as control cells. In vivo, in bone marrow transplantation assays, expression of sPRDM16 in a wild type background induces AML with low penetrance. Instead, sPRDM16 expression in a p53-/- background induces AML characterised by trilineage dysplasia with a shorter latency and full penetrance. The leukaemia is transplantable into secondary recipient mice.

Therefore, only the PR domain-negative form of PRDM16 is oncogenic and contributes to leukaemia by inducing a stem cell phenotype. The mouse model we have created of AML caused by sPRDM16 reflects the human disease and will serve as a valuable tool for dissecting the molecular mechanisms underlying AML, as well as providing a model in which to test targeted therapies.
Embryonic Germ (EG) cells are pluripotent stem cells derived from Primordial Germ Cells (PGCs) and have been shown to contribute to all cell lineages of the developing embryo. During their development PGCs undergo imprint erasure and demethylation. Little is known about the differences in phenotype and developmental potential of EG cells derived from early PGCs, before germ cell migration and imprint erasure, and those derived from later stage PGCs, once the germ cells have reached the genital ridges and imprint erasure is almost complete. Even less is known about the differences between EG cells and embryonic stem (ES) cells, which are derived from the early epiblast. By comparing gene expression and developmental potential in the different pluripotent stem cell populations, any similarities in gene expression between the cells lines, all of which differ in their origin, may help in the understanding of the pluripotency state. Any differences may reflect changes in the epigenetic state from epiblast to newly established PGCs to post migratory PGCs. As ES cells are quite unstable epigenetically, it will be important to know and understand how these developmental epigenetic changes are regulated.
The pluripotent nature of embryonic stem cells (ESC) has created an increasing demand to translate the therapeutic potential of these cells to a clinical application. However, the clinical application of these cells is hindered not only by ethical hurdles, but by their ability to form teratomas and immune rejection upon transplantation. Like ESCs, embryonic germ cells (EGC) have been derived via culture induced reprogramming, however propagation and use of human fetal material has prevented the therapeutic development of EGCs. Recently, studies have demonstrated that the elasticity of the germ-line to undergo culture induced reprogramming is not exclusive to the pre-natal environment but extends to the post-natal environment. One study reported the derivation of ES-like cells from neonatal mice that expressed pluripotent markers, contributed to chimeric cell populations when injected into blastocysts, and generated teratomas. Studies by us and a German laboratory have demonstrated that pluripotent cells can also be derived from adult mice, which demonstrate the efficacy of culture-induced reprogramming of the post pubescent germ-line. In this report, we used a transgenic mouse model expressing Oct-4-GFP as a reporter marker to track adult germ cell (AGC) derivation. We found that growth factor reprogramming is a dynamic process involving down and up-regulation of Oct-4 expression. Furthermore, our AGCs are similar, but not identical to ES-like cells because AGCs produced chimeric mice but did not form teratomas. In addition we report that adult human germ cells can also be reprogrammed to become AGCs that are characteristically similar to murine AGCs, including Oct-4 up-regulation, pluripotent marker expressions (Nanog, SSEA-4, TRA-1-60, Rex-1 and Alkaline phosphatase), and the ability to undergo spontaneous and induced differentiation into cardiomyocytes, neural cells, chondrocytes and adipocytes. Thus, therapeutically reprogrammed adult germ cells can become a novel pluripotent stem cell source for use in autologous cell based regenerative medicine.
Ruth Slack

Abstract P73

p107, a Retinoblastoma family member, regulates neural precursor cell division and differentiation by repressing Hes1

Jacqueline L. Vanderluit, Crystal Wylie, Kelly McClellan, Andre Fortin, Steve Callaghan, Jason G. MacLaurin, David S. Park, and Ruth S. Slack

University of Ottawa, Department of Cellular and Molecular Medicine, Ottawa Health Research Institute - Neuroscience Program, 451 Smyth Rd, Ottawa, Ontario, Canada K1H 8M5

The retinoblastoma (Rb) family proteins are key cell cycle regulators. While many studies suggest that p107 and pRb have overlapping functions, biochemical analyses reveal that they exhibit distinct binding preferences and different expression patterns. For example in the developing brain, p107 is specifically expressed in the proliferating cells surrounding the ventricular zone while pRb is expressed uniformly throughout the tissue. We have previously shown that p107 negatively controls both the number of neural precursors and their capacity for self-renewal. Here we show that the expanded proliferating neural precursor population in p107 deficient mice is at the expense of neural differentiation, such that during embryonic development differentiation of cortical neurons is delayed in p107 deficient mice. Since the Notch-Hes signaling pathway has been shown to regulate the size of the neural precursor pool and p107 deficient mice exhibit enhanced expression of Notch1 and Hes1; we questioned whether this pathway is responsible for the increased neural precursor population and differentiation delay in p107 null mice. To address these questions, we interbred p107 and Hes1 deficient mice to generate double null mutants. Loss of Hes1 in p107 null embryos restored the number of neural precursors to wild type levels. In addition loss of a single Hes1 allele reduced the number of proliferating progenitor cells and partially restored the differentiation defect in p107 null mice demonstrating that the Notch-Hes pathway also regulates this population. Deregulation of Hes1, therefore accounts for the enhanced numbers of neural precursors in both embryonic and adult p107 null mice. Luciferase reporter assays demonstrated robust repression of Hes1 promotor activity by p107. These results reveal an interaction between the cell cycle inhibitor, p107, and the Notch-Hes signaling pathway that directly impacts neural precursor numbers. In summary, we show a novel mechanism for p107, whereby repressing Hes1, p107 not only negatively regulates neural precursor self-renewal but also promotes differentiation.

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Natalia Slepko

Abstract P74

Expansion and Differentiation of Reprogrammed Adult Human Germ Cell-Derived Stem Cells after Optimization of Adherent Culture Conditions

Natalia Slepko, Francisco Silva, K.-Y. Francis Pau, Fari Izadyar, Chad Maki, Kyle Howerton, Jane Pham, Jason Pacchiarotti, Thomas Ramos, Joel Marh, and Chauncey Sayre

PrimeCell Therapeutics LLC, 213 Technology Drive, Suite 100, Irvine, California 92618, USA

We have reported previously that culture-induced reprogramming of mouse post-natal germ cells generates pluripotent germ line stem cells that, like embryonic stem cells, can populate tissues from all three germ layers during fetal development in vivo. To translate these mouse studies to human, we have developed methods for isolation, enrichment and culture of adult human germ cell-derived stem cells (AHGSCs) and found that the PM-10 medium formulated in our laboratory is an effective medium for cell growth and reprogramming. In this report, we focused on adherent culture in combination with addition of serum or serum replacement in the PM-10 medium for expansion and characterization of reprogrammed AHGSCs. Adult HGSCs were isolated after enzymatic dissociation and differential adhesion, plated onto fibronectin or gelatin substrates and cultured in the reprogramming PM-10 medium containing fetal bovine serum (FBS, 20%) or serum replacement (20%). These cultures were propagated for over 14 passages. Characterization of reprogrammed AHGSC populations showed that they (1) retained normal karyotype (46 XY) at passage 5, (2) expressed stem cell markers such as Oct4, Nanog, Sox2, Rex1, SSEA-4, and (3) exhibited markers of all three germ layers (Vimentin, Brachyury, alpha-fetoprotein). Moreover, AHGSCs retained their developmental potential and differentiated, upon exposure to specific conditions, into phenotypes exhibiting neuronal (including MAP-2 and NF-60), glial (including GFAP and MBP), cardiocyte (including cardiac actin and myosin), chondrocyte (including Collagen Xα1 and Perlecan), osteocyte (including Osteocalcin and Osteopontin), adipocyte (Oil Red staining), and endodermal lineage (including Albumin and HNF-3β) cell markers. These results suggest that AHGSCs after reprogramming and expansion in culture conditions based on the PM-10 medium retain karyotype normalcy and differentiation potential that can be further developed into a multipotent cell source for therapeutic applications in regenerative medicine.
Karel Smetana, Jr.

Abstract P75

Niche-like properties of cells originating from stroma of basal or squamous cell carcinoma

Karel Smetana, Jr., Barbora Dvorankova, Lukas Lacina, Zdenek Cada, Martin Chovanec, Sabine Andre

Charles University, 1st and 2nd Faculty of Medicine, Institute of Anatomy and Center of Cell Therapy and Tissue Repair, Department of Dermatoenerology, Department of Otorhinolaryngology, Head and Neck Surgery, Prague, Ludwig-Maximilians University

Tissue stem cells can play a role in solid cancer formation. Nonmalignant stem cells require special microenvironment-niche for maintenance of their stemness. Such an activity can be provided by stromal cell of tumor, where examples indicate effect of stroma on biological properties of tumor. This study summarizes data about influence of stroma cells prepared from basal or squamous cell carcinoma on phenotype and function of normal interfollicular keratinocytes.

Fibroblasts (Kerat-Vim+) isolated from both tumor types are able to change the shape, growth characteristics and phenotype of cocultured interfollicular keratinocytes in comparison with cells cultured in the presence of normal feeder fibroblasts. Presence of tumor fibroblasts induces expression of both the K8 and K19 in normal epidermal keratinocytes and strong expression of nucleostemin. These keratinocytes present beta-catenin in their cytoplasm which contrasted with predominant expression of this protein in intercellular contacts of keratinocytes cultured with normal feeder. Akin to cells of the squamous cell carcinoma FaDu line, keratinocytes cocultured with tumor fibroblasts coexpress both the vimentin and keratins indicating a high extent of epithelial-mesenchymal transition. To evaluate the role of direct contacts of keratinocytes with tumor fibroblasts, an insert system where both cell populations were separated by microporous membrane, was also used leading to very similar results. Cultivation of tumor fibroblasts with normal keratinocytes in 3-D system using Matrigel also demonstrated a significant influence of tumor stroma cells on normal keratinocytes. The presented observations reveal a high protumorigenic activity of tumor stromal cells and support the hypothesis about niche-like properties of tumor stroma.

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Reversion to a diploid state following ES-somatic cell fusion

Huseyin Sumer, Karen Jones, Antonius van Boxtel, Daniele Pralong, Paul Verma.

Monash Institute of Medical Research, Monash University, 27-31 Wright Street, Clayton VIC 3168, Australia

Difficulties associated with human SCNT/therapeutic cloning emphasize the relevance of using ES cells to reprogram somatic cells by cell fusion. Both mouse (Tada et al., 2001) and human ES cells (Cowan et al., 2005 and Yu et al., 2006) have been shown to reprogram certain aspects of gene expression in somatic cells following cell fusion; however the resulting hybrid cells are tetraploid and therefore have limited therapeutic application. We have developed a method of cell fusion that overcomes this limitation and have previously shown a proof of principle with a reversion to a diploid cell following mouse ES-ES cell fusion (Pralong et al., 2005). In the present study we demonstrate cell fusion between mouse ES and somatic cells (mesenchymal stem cells) and enrichment for heterokaryons (cells containing separate nuclei and a common cytoplasm). Further we maintain heterokaryons in culture for 48hrs and subsequently show selective post-fusion elimination of the ES nucleus. This method will allow for the examination of interactions between ES and somatic nuclei in fused cells and provides a means for reversion of ES-somatic heterokaryons to a diploid state.

References
A novel function of calreticulin as an inhibitor of adipogenesis

Eva Szabo, Marek Michalak and Michal Opas

Laboratory Medicine and Pathobiology/Institute of Medical Sciences, University of Toronto, Toronto, Ontario, Canada M5S 1A8; Membrane Protein Research Group and Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2H7.

Calreticulin is an ER-resident protein, which through its three distinct domains, N, P and C domains affects numerous cellular functions, such as chaperoning [N+P] or calcium storage capacity [P+C]. Calreticulin has been shown to be a negative transcriptional regulator of peroxisome proliferator-activated receptor (PPAR), by inhibiting of PPAR/RXR (retinoic acid receptor) binding to peroxisome proliferator-responsive DNA elements (PPRE). Additionally, calreticulin was shown to bind GC-rich stem-loop structures of CCAAT/enhancer-binding protein (C/EBP) mRNA, thus inhibiting C/EBP translation. C/EBPα together with PPARγ2 are thought of as the two most important transcription factors needed for adipogenesis. Here we show that calreticulin plays a role in adipogenesis, as its presence inhibits expression of PPARγ2, C/EBPα and aP2, a late adipogenic marker. Calreticulin (crt) expressing (+/+) WT ES cells and L7crt+/− cells (expressing crt flanked by loxP sites and a pANMerCreMer vector), showed a marked reduction in adipogenesis, as indicated by decreased staining with Oil Red O and attenuated expression of adipogenic markers. On the other hand, crt−/− cells, and L7crt−/− cells (with crt ablated by the induction of Cre recombinase with tamoxifen), exhibited a significant increase in adipocyte differentiation by the same standards. Increasing cytosolic [Ca2+] inhibited adipogenesis ongoing in the crt−/− cells. Decreasing cytosolic [Ca2+] increased adipogenesis in crt+/+ cells and had no effect on the crt−/− cells. Next, we expressed either the [N+P] domains or [P+C] domains of crt in crt−/− cells to restore the chaperone and calcium storage functions of calreticulin, respectively. The crt−/− cells containing the [N+P] domains differentiated into adipocytes, while the crt−/− cells containing the [P+C] P domains did not. Thus, the effect of calreticulin on adipogenesis is exerted through its functions in cell calcium homeostasis.

Two signalling pathways, calmodulin/CaMK II and ERK, were examined next in order to determine how calreticulin exerts its effect on adipogenesis. Both pathways were found to be activated in crt−/− cells, compared with the crt+/+ cells. Calmodulin inhibition (by W7) increased adipogenesis in crt+/+ cells, while inhibition of CaMK II (by KN-62) halted adipocyte differentiation in crt−/− cells.

In conclusion, this is the first study demonstrating that calreticulin is a negative regulator of adipogenesis that exerts its inhibitory effects at early stages of adipocyte differentiation by regulation of the calcium-dependent signalling pathways.
Mutations in the human MECP2 gene lead to a devastating neurodevelopmental disorder, Rett syndrome. However, the molecular etiology mediated by MeCP2 mutations remains largely unknown partly due to a lack of thorough understandings in MeCP2-regulated cellular processes. Transgenic mice deficient in MeCP2 can recapitulate a spectrum of symptoms observed in human patients, suggesting a functional and genetic link between MeCP2 and neuronal maturation processes in the postnatal brain development. MeCP2 belongs to a family of Methyl-CpG binding proteins, which are characterized by their DNA methylation-dependent DNA binding abilities. Previous biochemical analyses demonstrated that MeCP2 can associate with transcriptional repressor/co-repressor complexes mSin3A/HDAC1 or REST/CoREST. Based on existing genetic and biochemical evidences, MeCP2 is hypothesized to transcriptionally regulate genes that are critical to neuronal maturation and/or synaptic functions. Efforts to identify MeCP2 target genes, however, had limited success. Most notably, expression profiling of RNAs from MeCP2<sup>+/y</sup> and MeCP2<sup>−/y</sup> mice failed to reveal significant gene expression changes. More recently, using a candidate gene approach, our lab and Dr. Greenberg’s group identified the first neuronal target of MeCP2 in embryonic cortical neurons, Bdnf. Since then, several target genes are identified by screening upregulated genes in mice lacking MeCP2, including Dlx5 and several glucocorticoid-regulated genes. However, none of these genes have been shown to be direct functional target genes involved in Rett syndrome etiology. Our ability to take an unbiased approach to identify MeCP2 targets is partly hindered by the complicated cellular composition in mammalian central nervous system. In order to overcome these difficulties, we developed a differentiation procedure to derived subtype-specific neurons with high purity (>90% GABAergic) and in large quantities from both wild-type and MeCP2 deficient embryonic stem cells. By RT-PCR and immunostaining using lineage and regional specific markers, we showed that these GABAergic neurons are of midbrain origin. We plan to exploit this in vitro culture paradigm to identify MeCP2 targets by microarray-based expression profiling. With identifications and functional validations of MeCP2 targets in well-defined, homogeneous neuronal populations, we believe that new insights will be provided to design therapeutic strategies for Rett syndrome.
Aaron Thorner

Abstract P79

Isolation and Characterization of Human Mammary Stem Cells

Aaron R. Thorner¹, Olga Karginova², Katherine A. Hoadley¹, and Charles M. Perou¹,²,³

¹ Curriculum in Genetics and Molecular Biology, ² Lineberger Comprehensive Cancer Center, ³ Departments of Genetics and Pathology and Laboratory Medicine, University of North Carolina, Chapel Hill, NC, USA

Somatic stem cells constitute 1-2% of all cells in a mature organ system and are capable of undergoing self-renewal and multi-lineage differentiation. It is becoming widely accepted that at least a subset, if not most, cancers arise from stem cells. Since stem cells are long-lived, there is also a greater potential to accumulate multiple genetic hits leading to cellular transformation and tumorigenesis. Thus, the study of adult stem cells is necessary for both an understanding of normal organ development as well as carcinogenesis.

The human mammary glands consist of a series of hollow, branched ducts that are comprised of luminal epithelial cells, which line the inside of the ducts, and myoepithelial cells, which surround the luminal cells and produce the basal lamina. A somatic stem cell with the ability to rapidly generate these distinct cell types must reside within the mammary gland and significant progress has recently been made in identifying these cells in mice (Stingl et al., 2006; Shakleton et al., 2006). We are focused on isolating and characterizing human mammary stem cells, and defining their relationship relative to the breast tumor “intrinsic” subtypes that have been defined using gene expression profiling (Hu et al., 2006). We are using two complementary approaches, the first of which is a cell culture assay that enriches for undifferentiated progenitor cells from reduction mammoplasty tissues (Dontu et al., 2003), and the second is FACS. In addition, we are testing the ability of these potential stem cell enriched populations to undergo multi-lineage differentiation using in vivo and in vitro differentiation assays and DNA microarray analyses. Our preliminary results using mammospheres have recapitulated the finding that these cells can differentiate from originally Keratin-negative cells into Keratin 5/6 and Keratin 8/18 positive cells. However, when assessed by gene expression profiling, a robust luminal/ER+ profile was not observed suggesting that differentiation into a fully mature luminal cell has not occurred. Lastly, genomic analyses of antibody-mediated cell sorted populations will also be presented.
Isolation, characterization and differentiation of Mesenchymal Stem Cells derived from adult adipose tissue

Trejo C¹, Gómez-Gil V², Corrales C², Pascual G², García-Honduvilla N¹, Buján J², Bellón JM¹.

Centre: Depts. of ¹Surgery and ²Medical Specialties, Faculty of Medicine, Univ. of Alcalá de Henares, Madrid, Spain.

Introduction. Mesenchymal stem cells derived from adult adipose tissue (AD-MSC) have the capacity to differentiate into different cell types. Given that these cells could have applications in wound repair, the aim of this study was to characterize and differentiate this cell population. Materials and methods. AD-MSC isolated from the subcutaneous fatty tissue of Wistar rats (n=5), were maintained in culture under standard conditions for over a year. Immunohistochemical techniques were used to establish the expression of several markers (Nanog, Oct 3/4, CD9, SSEA-1, E-caderin, M-caderin, P0DXL, SSEA-4, vimentin, desmin, fibronectin, integrin β¹, α actin and CD34) and proliferation assays were undertaken by flow cytometry. Data analysis was performed using Fisher’s exact test. The cells were differentiated into adipogenic and osteogenic lineages by adding specific supplements to the culture media and then subjected to immunohistochemical, histochemical and enzyme analyses.

Results. AD-MSC could be readily maintained in vitro and were subcultured over a hundred times with no effects on growth rate or cell morphology. Our proliferation studies indicated that after three days of culture, cell numbers increased significantly from one time point to the next. Cell counts at 15 days were 2.06 x10⁶ cells/ml (206 times the starting concentration). DNA assays revealed a predominance of G2/M stage cells in the cultures. Throughout the study period, the AD-MSC expressed Oct3/4, SSEA-1, E-caderin, P0DXL and vimentin; desmin expression was not observed until the 48th subculture. In cultures passaged over 125 times, expression levels of Nanog, CD9, M-caderin and integrin β¹; increased and SSEA-4 expression diminished and became negative. CD34, fibronectin and α actin were only expressed by a proportion of the cell population, which was similar in all the subcultures. Cells were positive for markers of differentiation towards both the osteogenic lineage, identified by von Kossa, alizarin red and alkaline phosphatase staining, and the adipogenic lineage, revealed by the oil red test.

Conclusions. Cells isolated from the subcutaneous tissue of the rat and then cultured show the typical characteristics of undifferentiated pluripotent mesenchymal cells. Our findings confirmed the capacity of these cells to differentiate into adipogenic and osteogenic cell lineages.
Terumasa Umemoto

Abstract P81

The properties of quiescent hematopoietic stem cells were correlated to expression of CD61 (integrin beta 3)

Terumasa Umemoto, Masayuki Yamato, Yoshiko Shiratsuchi, Masao Terasawa, Joseph Yang, Kohji Nishida, Yoshiro Kobayashi and Teruo Okano

Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan.
Division of Molecular Medicine, Department of Biomolecular Science, Toho University, 3-3-1 Miyama

With significant attention paid to the field of tissue-specific stem cells, the identification of stem cell-specific markers is of considerable importance. Previously, the side population (SP) phenotype with the capacity to efflux the DNA-binding dye Hoechst 33342, has been recognized as a common feature of adult tissue-specific stem cells. Here, we show that high expression of CD61 (integrin beta 3) is a common attribute of SP cells isolated from both the mouse bone marrow and rabbit limbal epithelium. Additionally, using the hematopoietic system, we confirmed that the expression of CD61 is correlated with properties of quiescent hematopoietic stem cells (HSCs) including SP phenotype, cell cycle arrest, expression of HSC markers and long-term hematopoiesis. Importantly, Lineage- (Lin-)/ CD61High SP cells have a stronger capacity for long-term hematopoiesis than even c-Kit+/ Sca-1+/ Lin- (KSL) SP cells, which are regarded as one of the most highly enriched HSC populations. Finally, CD61 subunit that presented in SP cells having HSC properties is associated with CD51 (integrin alpha V). Our results therefore demonstrate that high expression of CD61 is correlated the properties of stem cells, and suggested that the CD61 subunit is available as a common surface marker of tissue-specific stem cells.
Patrick Viatour

Abstract P82

Critical role of the RB gene family for the maintenance of Stem Cell quiescence

P. Viatour, IL. Weissman, E. Passegue and J. Sage

Department of Pediatrics, Rm 1200, CCSR South, 365, Pasteur Drive, Stanford University, Palo Alto, CA, USA

Rb was the first tumor suppressor gene to be identified, twenty years ago. Since then, the Rb signaling pathway has been shown to be inactivated in nearly all adult cancers. While Rb is known to play a critical role in the control of cell cycle, differentiation and apoptosis, the specific mechanisms underlying the tumor suppressor activity of Rb are still poorly characterized. In particular, the cell types in which loss of Rb function initiate cancer are not known. Another layer of complexity is added by the existence of p107 and p130, two proteins structurally and functionally related to Rb. While the three family members exhibit specific functions, they also share numerous cellular roles and often functionally compensate for each other’s absence.

In adult cancers, several events lead to the concomitant loss of function of the three members of the Rb family, such as decreased expression of p16 through promoter methylation, overexpression or increased activity of the cyclins/CDK kinases, or infection by oncoviruses such as HPV. In order to recapitulate this simultaneous loss of function of Rb family members, and to bypass the lethality of Rb family mutant embryos, we have used conditional alleles for Rb, p130 to generate Rb family conditional triple knock-out mice. Activation of Cre in cells from these mice results in the inactivation of the entire Rb family.

We will present evidence that the acute loss of function of Rb, p107, and p130 in adult mice leads to a general proliferative phenotype. More specifically, this inactivation of the entire Rb gene family is sufficient to initiate cancer in several organs and tissues, including in the hematopoietic compartment. This phenomenon originates from the expansion of mutant stem cell and progenitor cell populations. These results indicate that Rb family members normally play a central role to maintain adult stem cells and progenitor cells in a quiescent state in vivo and that disruption of this quiescent state is a key event in cancer initiation.
Yuko Wakamatsu

Abstract P83

Reprogramming of adult somatic cell nuclei of fish, medaka (Oryzias latipes), by a novel method of nuclear transfer using diploidized eggs as recipients

Ekaterina Bubenshchikova¹, Elena Kaftanovskaya¹, Nami Motosugi¹, Takaumi Fujimoto², Katsutoshi Arai², Masato Kinoshita³, Kenjiro Ozato¹ and Yuko Wakamatsu¹

¹Laboratory of Freshwater Fish Stocks, Bioscience and Biotechnology Center, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8601, ²Graduate School of Fisheries Sciences, Hokkaido University, Hakodate 041-8611, ³Division of Applied Biosciences, Graduate School of Agriculture, Kyoto University, Kitashirakawa-Oiwake-cho, Sakyo-ku, Kyoto 606-8502, Japan.

Despite several decades of work, reprogramming of adult somatic cell nuclei to pluripotency has been unsuccessful in non-mammalian animals. The main reason is chromosomal aberrations in reconstructed individuals, which are caused by asynchrony between the cell cycles of the recipient egg and donor nucleus. To date, nuclear transfers have been conducted using enucleated eggs as recipients. In a fish, the medaka (Oryzias latipes), we tried a novel method, using diploidized eggs as recipients, to normalize chromosome status instead of using enucleated eggs. We transferred the nuclei of primary cultured cells from the caudal fin of adult green fluorescent protein (GFP)-transgenic fish to eggs that had been made diploid by retention of the second polar body release. Via this process, the one cell stage-reconstructed embryo contained two diploid nuclei, one from the recipient egg and one from the donor cell, in the same cytoplasm. We found that 2.7% of the reconstructed embryos that started to develop became adults that expressed GFP in various tissues in the same pattern as that in the donor fish. Moreover, these fish were diploid, fertile and capable of passing the marker gene on to the next generation in a Mendelian fashion. Thus, the successful nuclear transplants were similar in genotype and phenotype to the donor. We hesitate to call these individuals clones because we used non-enucleated eggs as recipients and, thus, they may be chimeras that consist of cells derived from both the diploid recipient and donor nuclei. In any case, fish adult somatic cell nuclei were reprogrammed to pluripotency and differentiated into a variety of cell types by using diploidized recipient eggs. This novel method may prove an effective way to overcome cell cycle asynchrony and contribute to a broader understanding of the nuclear reprogramming of vertebrate cells.
Stable shRNA expression in primary cells has proven to be a challenge. Therefore, we sought a simple retrovirus (MSCV) system to deliver shRNA. Here we show that MSCV-LMP is capable of stable shRNA expression targeting SHIP-1 in murine peripheral cells originating from transduced hematopoietic stem cells. In addition to decreased SHIP-1 intracellular staining, peripheral B cells positive for a surrogate marker of SHIP-1 knockdown (GFP) exhibit increased migration to the chemokine SDF-1α similar to that seen with B cells from SHIP-1−/− mice. We conclude that stem cells transduced with MSCV-LMP expressing SHIP-1 shRNA can reconstitute the peripheral lymphoid compartment while maintaining SHIP-1 knockdown.
Amanda Waterstrat

Abstract P85

Genetic Regulation of Hematopoietic Stem Cell Number

Amanda Waterstrat¹, Ying Liang¹, Hartmut Geiger³, Hua Li², Arnold Stromberg², Gary Van Zant¹

¹University of Kentucky Markey Cancer Center, USA
²University of Kentucky Department of Statistics, USA
³Cincinnati Children’s Hospital Medical Center, USA

Endogenous stem cell number is a complex, quantitative trait that varies widely among individual humans and among inbred strains of mice. In vitro quantification of hematopoietic stem cells (HSCs) coupled with linkage analysis in C57BL/6, DBA/2 and a panel of BXD recombinant inbred strains revealed that loci on at least 5 chromosomes are linked to the number of HSCs in the bone marrow of young adult mice. Given the numerous genetic similarities between mouse and human, our goal is to identify genetic regulators of HSC number in the mouse that will reveal regulatory mechanisms governing HSCs in humans and thus provide an avenue for clinical intervention in the treatment of hematopoietic malignancy and other diseases originating in the HSC compartment.

Young C57BL/6 mice possess at approximately three-fold more hematopoietic stem cells per femur than young DBA/2 mice, as measured by the Cobblestone Area Forming Cell (CAFC) assay. In order to confirm that the QTL identified on mouse chromosome 5 significantly influences HSC number, we quantified HSCs in mouse strains congenic for the chromosome 5 QTL. The congenic mouse strains possess the genome of one parental strain with the exception of a roughly 30 cM interval on chromosome 5 bearing the genotype of the opposite strain. In these strains, the chromosome 5 QTL was sufficient to confer a phenotype that was either increased or decreased from the level of the background strain toward the phenotype of the strain represented in the congenic interval.

Having established the functional significance of the QTL region, we are currently characterizing potential candidates using a microarray-based approach. Total RNA isolated from stem and progenitor cells of C57BL/6, DBA/2 and congenic mice was used for transcript profiling on the Affymetrix 430v2 and Illumina Sentrix Mouse-6 whole-genome microarray platforms. Preliminary results reveal that approximately forty-one transcripts within the congenic interval are differentially expressed in one parental-congenic strain comparison. Three of these genes are upregulated in mice with higher stem cell numbers. We are currently determining whether or not the expected patterns of gene expression exist in the second parental-congenic strain pair and confirming transcriptional differences of QT gene candidates using RT-PCR. Thus far, transcript profiling supports the assumption that a gene on mouse Chromosome 5 is a positive regulator of HSC number in the mouse.
Hao Wu

Abstract P86

Dnmt3a-G9a complex represses differentiation genes to maintain self-renewal of neural stem cells

Hao Wu1, Volkan Coskun1, En Li2, Guoping Fan2, Yi Eve Sun1

1 Departments of Molecular & Medical Pharmacology and Psychiatry & Behavioral Sciences, MRRC at UCLA Neuropsychiatric Institute
2 Department of Human Genetics, UCLA School of Medicine, NRB room 351, 635 Charles E. Young Drive South, Los Angeles, CA 90095

Mechanisms by which stem cells self-renew while maintaining their differentiation potential are linked to heritable epigenetic modifications of differentiation genes. The repressed yet poised chromatin structure of key developmental genes in embryonic stem cells (ESCs) is partly maintained by polycomb group (PcG) complexes, and is required for the maintenance of pluripotency. However, PcG-mediated repression is released during ESC differentiation, suggesting that additional epigenetic machineries mediate repression of poised differentiation genes in self-renewable tissue-specific stem cells. Here we report that during the transition from ESCs to multipotent neural stem/progenitor cells (NPCs), repressive epigenetic marks in poised glial differentiation genes switch from PcG-mediated tri-methylation of lysine 27 on histone H3 (H3K27me3) to di-methylation of lysine 9 on histone H3 (H3K9me2); this switch is associated with increased DNA cytosine methylation by the de novo DNA methyltransferase Dnmt3a. Surprisingly, Dnmt3a reversibly represses genes by DNA methylation and by a novel, DNA methylation independent mechanism that involves recruitment of the histone H3-K9 methyltransferase G9a. Deficiency in Dnmt3a or G9a disables the self-renewal capacity of NPCs and causes their spontaneous differentiation into glial cells; in Dnmt3a-null NPCs, this phenotype is partially rescued with an enzymatically inactive Dnmt3a mutant that still interacts with G9a. Our data thus indicate that the Dnmt3a-G9a complex establishes repressive marks on poised glial lineage genes to maintain NPCs in an undifferentiated state, suggesting that reversible repression of poised differentiation genes is a common epigenetic strategy for stem cell maintenance, but mediated by distinct epigenetic machineries in ESCs and somatic stem cells.
Identification of cardiomyogenic markers in porcine mesenchymal stem cells.

Ybarra N¹, Vincent P¹, Smith CL¹, Jankowski M², Gutkowska J², Gauvin D¹, Troncy E¹.

¹ Department of veterinary biomedicine, Faculty of veterinary medicine – Université de Montréal.
² Centre Hospitalier de l’Université de Montréal – Research Center.

Cell therapy is becoming an alternative treatment for replacement of dead myocardium after ischemic insult. Mesenchymal stem cells (MSC), derived from bone marrow, are undifferentiated cells with self-renewal capacity for long periods. MSC have myogenic differentiation potential and when delivered to the infarct area, they release cytokines and growth factors that stimulate endogenous repairing mechanisms. Expression of transcription factors and structural proteins by MSC is altered with the number of passages. It has been shown that MSC have passage-restricted differentiation potential. Swine is a more suitable model for myocardial infarction, due to similar physiological and anatomic characteristics with humans. Therefore, in this study we attempt to characterize the optimum time of porcine MSC differentiation based on expression of some transcription factors and structural proteins expressed at different passages. MSC were obtained from bone marrow aspirates. Mononuclear cells were seeded. When homogenous colonies were observed, cells were characterized by surface markers expressed in MSC, using FACS analysis. The expression of transcription factors and structural proteins was also assessed by RT-PCR. FACS analysis revealed that porcine MSC were CD90, CD29 positive and CD45 and CD31 negative. RT-PCR showed expression of transcription factor Oct-4, used as a marker for undifferentiated, pluripotent cells, GATA-4 transcription factor expressed in early embryonic cardiogenesis, phospholamban, cardiac structural protein, and oxytocin receptor (OTR). Expression of these markers was observed only during passages 0 and/or 1 and disappeared or was down-regulated in subsequent passages. MSC did not express other cardiac cell markers, like cardiac myosin heavy chain or atrial natriuretic peptide.

With regards of these results and the previous demonstration of the role of oxytocin (OT) as a potent and specific differentiation inducer of murine embryonic and cardiac stem cell into beating cardiomyocytes, it looks appropriate to test in vitro OT-induced differentiation of porcine MSC into cardiomyocytes during the early passages.

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Effect of hypoxia on the transcriptional signature of mouse ATDC5 cells during chondrogenesis

Vladimir Zachar, Li Chen, Trine Fink, and Peter Ebbesen

Laboratory for Stem Cell Research, Aalborg University, Fredrik Bajers Vej 3B, 9220 Aalborg, Denmark

The morphogenesis of cartilage occurs in vivo under conditions that are characterized by low oxygen partial pressure. To obtain better understanding of the role of hypoxia in this process, the mouse chondroprogenitor cells ATDC5 were induced to differentiate by the addition of insulin and cultured under ambient and hypoxic conditions corresponding to 21% and 1% O(2) in the gas phase, respectively. The transcriptional profile of 104 selected genes was determined by semiquantitative real-time RT-PCR. Although, the hypoxia alone induced early chondrogenesis as evidenced by the expression of aggrecan and collagen type II genes, the hypoxic incubation of insulin-treated cells surprisingly delayed and suppressed the insulin-mediated early chondrogenesis and almost completely blocked hypertrophic differentiation. The stage-specific expressional patterns hinted to the mechanisms possibly involved. In addition, a group of genes was identified that have not previously been associated with hypoxia, including Ak4, Akt3, Col X, Fmod, Ier3, IGFbp4, MafF, Mxi1, Rcor2, Rras, Sox6, Tnni2, Wnt5a, and Zfp313.
Enrichment of and Characterization of Highly Tumorigenic Cells with Cancer Stem-Cell Properties from Human Glioma Cell Line U87

Yunfei Zhou*, Yan Zhou*, Li Feng*, Peng Huang* ^

* Department of Molecular Pathology, Unit 951, University of Texas, M. D. Anderson Cancer Center, Houston, TX 77230-1429, USA. ^ Corresponding author

Significance: In this study, we used human glioma cell line U87 as a model system to demonstrate that the established cancer line may contain subpopulation of cells with cancer stem-cell properties, which are tumorigenic, can be enriched \textit{in vivo}, and subsequently can be maintained at the stem-cell stage under proper culture conditions. This study provides new insights into cancer cell biology and may serve as a useful model to study cancer stem cells.

Methods: The human malignant glioma U87 cells were routinely maintained in DMEM medium containing 10% FBS. Cells were injected subcutaneously into nude mice to establish xenograft tumors. Three weeks later, cells were isolated from the freshly dissected tumor mass following standard primary cell culture procedures. The isolated cells, designated as U87-SC, were maintained in a simplified brain tumor stem cell culture medium (BTSC medium) containing 20ng/ml EGF and 5% FBS under normoxia or hypoxia (2% O_2) conditions. Single neurosphere-like cancer cell clusters were further isolated and expanded in BTSC medium. Cell morphology and the ability to form neurospheres and to undergo differentiation were tested. Neural stem cell markers (nestin, CD133, ABCG2) were characterized. Tumorigenicity of the U87-SC cells and the parental U87 cells was quantitatively tested in nude mice xenograft model, by monitoring tumor forming at various inoculation densities ($10^3$-$10^6$/mouse) on the left and right flanks of the same mice, respectively.

Results: U87-SC cells cultured in BTSC medium (5% FBS + EGF) readily formed neurosphere-like cancer cells cluster at earlier passages under hypoxia and normoxia, with hypoxia significantly favored the maintenance of neurosphere-forming capacity. U87-SC cells growing in regular medium + 10% FBS rapidly lost the neurosphere-forming phenotype both in normoxia and hypoxia. Neurospheres derived from U87-SC are \textit{rich} in nestin, CD133, and ABCG2 positive cells. Interestingly, western blot analysis showed the neurosphere cells had an increased expression of nestin but not CD133. CD133 expression was upregulated in U87-SC but not U87 cells under hypoxic condition. Cell clone derived from a single sphere of U87-SC cells could be expanded in BTSC medium to form secondary neurospheres, which changed to monolayer cells when shifted to regular culture medium in normoxia condition. Neuron-differentiation phenotype can be induced from U87-SC cells after doxorubicin treatment, although the parental U87 cells also contain a smaller percent of cells that can be induced to differentiate. \textit{In vivo}, U87-SC cells were 10-100 fold more tumorigenic than the parental U87 cells.

Conclusion: Established human glioma U87 cell line contains a subpopulation of cells with cancer stem-cell characteristics, which can be enriched by \textit{in vivo} selection and maintained \textit{in vitro} under proper culture conditions. The cells in cancer stem-cell stage are highly tumorigenic, and the stem-cell stage can be readily shifted by culture condition.
Zhuo Zhou

Abstract P90

Analysis of -globin locus activation during erythroid differentiation of embryonic stem cells

Zhuo Zhou, Valerie Crusselle-Davis, Linda I-Chun, Padraic Levings, and Jorg Bungert

Department of Biochemistry and Molecular Biology, Genetics Institute, University of Florida, Gainesville, FL 32610

The -globin gene locus consists of five developmentally regulated genes that are under control of a locus control region (LCR) located far upstream of the genes. The LCR is composed of several subregions exhibiting high sensitivity to D Nasel in erythroid cells, called HS sites, which function together to mediate high level globin expression. Recent studies have shown that individual HS sites recruit transcription complexes. We found that RNA polymerase II (RNA Pol II) is recruited to LCR HS elements prior to erythroid differentiation. In order to understand the temporal order of transcription factor recruitment and modification of chromatin structure, we continued our analysis of the globin locus during in vitro differentiation using chromatin immunoprecipitation. Several transcription factors, including the ubiquitously expressed helix-loop-helix protein USF are detectable at LCR elements prior to erythroid differentiation. We have recently developed an in vitro system to study the transfer of activities from the LCR to the -globin gene. We found that RNA Pol II can be transferred from immobilized LCR constructs to a -globin gene, that the transfer is sensitive to mutations in the basal promoter of the -globin gene, and that the hematopoietic transcription factor NF-E2 facilitates the transfer from the LCR to the globin gene promoter. We continued this analysis and found that other proteins are transferred from the LCR to the globin gene promoter, including USF, Fog-1, and GATA-1. Our data suggest that NF-E2 decreases the affinity of RNA Pol II to the LCR and at the same time increases the binding affinity to the -globin gene promoter. Taken together our data support the proposition that transcription complexes are first recruited to the LCR and subsequently transferred to the globin genes.
Duchenne Muscular Dystrophy (DMD) is a lethal X-linked disorder characterized by progressive muscle wasting and weakness which affects 1 in 3000 male births. It is caused by mutations in the dystrophin gene resulting in total absence of the protein dystrophin in muscle fibers. To evaluate whether stem cell could contribute to skeletal muscle repair in vivo we used the canine model of DMD named Golden Retriever Muscular Dystrophy (GRMD) that presents a clinical progression comparable to the human form. Here, canine umbilical cord vein (cUCV) mesenchymal stem cells were isolated from normal Golden Retriever dogs. These cells grew forming fibroblastoid colonies and could be expanded successfully in culture. By immunocytochemistry, we showed that cUCV cells express the adhesion molecules CD29 and CD90. Their multilineage potential were assessed by inductive medium and analyzed by colorimetric tests, immunocytochemistry and RT-PCR. This is apparently the first study reporting mesenchymal stem cell isolation from dog umbilical cord vein. These cells represent a valuable tool for pre-clinical trials in the GRMD model and their skeletal muscle repair potential in vivo will be further analyzed. This work was supported by FAPESP-CEPID and CNPq.