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Receptor Control of Human Skin Cell Regeneration and Maturation

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Keratinocytes are the major cell type within the upper layer of skin. They must be replenished throughout our lifetime to compensate for wear-and-tear and wound healing. This regeneration occurs via stem cells, some of whose daughters retain replication capacity while others commit to a post-replication, endpoint. With a goal of identifying therapeutic compounds for skin maintenance and healing, we examined the effect of peroxisome proliferators (PPs) on human keratinocytes (KC). PPs are diverse chemicals including clinically approved drugs and fatty acids. They stimulate PP activated receptors (PPAR) leading to changes in gene expression. To date, PP treatment of human KCs in Petri dishes or mouse skin have established some effects on replication and maturation. However, Petri dish experiments are suboptimal for assaying the full range of possible KC responses. Additionally, rodent-specific PP effects exist that limit our full understanding of human KC responses. To address this, we investigated PP influence on human KC growth and maturation in organotypic cultures that recapitulate structural and biochemical markers of epidermis. We found PPARalpha agonists clofibrate and WY-14,643 and panPPAR agonist docasohexaenoate enhanced KC proliferation, increased the number of KC layers, and improved levels of the maturation-dependent proteins characteristic of fully functional skin. Some PP effects were distinct from those reported for rodent skin and Petri dish cultures suggesting species and growth context of KCs can impact exposure outcomes. Given the utility of organotypic cultures in modeling skin, these studies bridge the gap between rodent assays and clinical studies for capitalizing on replication enhancement of human KC stems cells in response to PPs.

Poster Board #1

Binucleation and Polyploidization in Human Exocrine Pancreas

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Objectives: Most mammalian somatic cells contain a 2N complement of DNA. However, in some cases, binucleation and incomplete mitosis give rise to polyploid cells, containing 4N or even higher multiples of the haploid genome. Well-known examples include megakaryotcytes and hepatocytes. While polyploidy is generally associated with functional maturation, the dynamic nature and functional significance of polyploidization in acinar cells of the exocrine pancreas are not well understood.
We previously demonstrated age-dependent polyploidization in acinar cells of the normal mouse exocrine pancreas. Here we demonstrate that polyploidization occurs in human pancreatic acinar cells as well, albeit at a lower percentage than that seen in mice.

Methods: Archival formalin-fixed tissue sections of human male pancreas were obtained through the Department of Pathology at Yale New Haven Hospital, with approval of the institutional Human Investigation Committee. Fluorescence in situ hybridization for the Y chromosome (Y-FISH) was used as a marker of genome number in pancreatic acinar cells. Immunostaining for E-cadherin was performed to delineate cell membrane boundaries. The number of nuclei per cell was quantitated by DAPI staining.

Results: Preliminary results indicate that binucleation was observed in up to 10 percent of all adult human male pancreatic acinar cells while greater than 10 percent of the cells contained a single polyploid nucleus. Similarly, exocrine cells from adult male mouse pancreas demonstrated greater than 10 percent binucleation and 40 percent polyploidization.

Conclusion: Binucleation and polyploidization are seen in adult human exocrine pancreas, at levels lower than that seen in adult mice. Future studies will address age and disease-related changes in acinar cell binucleation and polyploidization.

Adult Muscle Stem Cells and Their Precursors Express the Myogenic Determination Gene, Myod, During Embryonic Myogenesis and Postnatal Self-Renewal

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Satellite cells are tissue-specific stem cells that are responsible for postnatal skeletal muscle growth and repair. Although the developmental capacity of quiescent satellite cells has been controversial, our recent lineage analysis with a MyoD iCre driver and a Cre-dependent GFP reporter demonstrated that almost all adult satellite cells have expressed the myogenic determination gene, MyoD, in their developmental history. To address the timing of MyoD expression in satellite cell precursors, we generated a MyoD CreER knockin allele and performed conditional activation of Cre recombinase by stage-specific administration of tamoxifen. Tamoxifen administration at embryonic days 11 and 12 resulted in significant labeling of adult satellite cells, indicating a close lineage relationship with MyoD-expressing, lineage-restricted embryonic myoblasts and arguing against the prevailing view that satellite cells derive from a more primitive, uncommitted precursor. Administration of tamoxifen at postnatal days 6-10 also labeled adult satellite cells, suggesting that MyoD+ satellite cell precursors are born, or continue to express MyoD, over a broad developmental window. Further, tamoxifen-dependent recombination of satellite cells was observed among the self renewing population following cardiotoxin-induced muscle regeneration. Collectively, these data indicate that MyoD is involved not only in regulating muscle differentiation but also in myogenic commitment and maintenance of the satellite cell niche.

Poster Board #3

Myod: A Nodal Lineage for Skeletal Myogenesis

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Establishment of the myogenic lineage is regulated in a spatiotemporal manor by the coordinated expression of the muscle regulatory factors (MRFs), MyoD and Myf-5. Whereas embryos mutant for both genes completely lack skeletal muscle, single knockouts of MyoD or Myf-5 show only minor, transient muscle defects, indicating functional overlap between these transcription factors. Recent Myf-5 lineage ablation studies suggest there are two lineages of muscle precursor cells that lead to skeletal muscle differentiation; a Myf-5 dependent and independent lineage. In order to investigate the independence of the MyoD lineage from the Myf-5 lineage, we used Cre/lox lineage ablation of the MyoD lineage using Diphtheria Toxin subunit A (DTA). Prenatal embryos were analyzed by reporter gene expression, immunohistochemistry and in-situ analysis. Interestingly, we found that ablation of the MyoD lineage results in a complete loss of skeletal muscle, as well as satellite cell progenitors, as shown by the loss of Myosin heavy chain and Pax7 expression, respectively. While early myogenesis is unaffected at E10.5, by E12.5 a dramatic decrease in both myoblast and differentiation markers is detected and by E16.5 skeletal muscle is completely undetectable. Our experiments support the idea that all embryonic myogenic cells, including satellite cell progenitors, transit through a MyoD+ state, and that Myf-5-expressing cells represent a subpopulation of the MyoD-expressing lineage. Implications of MRF genetic redundancy and satellite cell programming will be discussed.

Mouse Embryonic Long-Label Retaining Cells in the Adult Mammary Gland

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Studies in several adult tissues have confirmed the presence of organ-specific stem cells that contain the capacity to proliferate and differentiate into all the lineages of a given tissue. Classical studies demonstrate that cells from mouse embryonic mammary rudiments have the capacity to generate an entire gland when transplanted into the cleared fat pad of adult recipient mice. However, no studies have examined the relationship of embryonic mammary cells to adult mammary stem cells. We were interested in determining whether embryonic cells only contribute to the initial formation of the ducts or whether they also contribute to the stem cells that are responsible for tissue homeostasis and that support the cyclical development of the mammary gland during reproductive cycles. Label-retaining cells (LRCs) have been the basis for identifying adult stem cell distribution in situ in many tissues, since stem cells are thought to cycle slowly and retain DNA labels for prolonged periods of time. We hypothesized that if embryonic cells contributed to the adult stem cell pool, then DNA labeling during the first phase of rapid ductal development and cell division (e15 to birth) might generate LRCs that would be retained in the developing ducts during puberty. We pulsed embryos using EDU labeling of proliferating cells during this phase, then chased these cells for up to 7-1/2 weeks after birth. Immunofluorescence was used to identify LRCs, as well as epithelial cells expressing the lineage markers, K14, Gata3 and P63, and the hormone receptor, PR. We found that 1% of the total cells retained EDU. The majority of LRCs were located in the basal compartment and expressed the myoepithelial lineage markers K14 and/or p63. Interestingly, LRCs were located only near the origins of the duct system and far from the terminal end buds, which serve as the active growth fronts of the ducts. We next performed sequential labeling with EDU at e15 to birth and with BrdU from the onset of puberty at 21 days for five days to label two different phases of active growth. These experiments demonstrated that label-retaining cells are found only at the site of active cell division at the time of DNA labeling. Furthermore, some of these LLRCs were mitotically active during puberty and in the adult gland as demonstrated by BrDU labeling. Thus, our data suggest that stem/progenitor cells in the mammary gland do not come from a subset of the embryonic cells that are carried forward with the growing ducts, but rather may be set aside at the site of active cell division and growth. Using FACS cell sorting, we found that a subset of these LRCs express cell surface markers previously described for mammary stem cells. We are currently investigating whether these LLRCs have function self-renewal characteristcs of stem cells. Future studies may be useful to help define the stem cell niche in the mammary gland and to determine how stem cells are set-aside during morphogenesis.

Identification of the Musculoskeletal Progenitor Niche

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Diseases of the mesodermal tissues of the body, which include heart disease and muscular wasting diseases, affect millions of people across the globe. These diseases could be treated with stem cell-based regenerative medicine if existing barriers to the efficient derivation of mesodermal tissues from embryonic stem cells (ESC) could be overcome. Robust directed differentiation of mesoderm is currently limited to cardiovascular and blood derivatives; reproducible protocols for the differentiation of muscle, cartilage, and connective tissues, or more specifically, those mesodermal derivatives produced solely from paraxial mesoderm, have not yet emerged.
Previous studies have indicated the presence of an endogenous stem cell located at the junction of the primitive streak and node, also known as the border. This cell is responsible for colonizing the paraxial mesoderm and giving rise to the somites which in turn produce the skeletal muscle, cartilage, and connective tissue of the embryo. This stem cell, therefore, is the precursor cell type for all axial muscle, cartilage, and connective tissue of the embryo posterior of the head. Characterizing the musculoskeletal progenitor (MS-Pr) niche and the MS-Pr itself would provide fundamentally important insight to the generation of musculoskeletal mesoderm during embryogeneis and would lay the foundation for the directed differentiation of muscle, cartilage, and connective tissue for regenerative medical treatment of musculoskeletal disease.
In this project we comprehensively characterize the PM-Pr and its niche and evaluate the role of key genes in maintenance of the MS-Pr and the MS-Pr niche *in vivo*. Successful completion of these goals will provide unprecedented insight to the generation of somitic mesoderm and enable novel approaches to the generation therapeutically relevant mesodermal cell types in vitro.

Ngn3 Programming of Endocrine Pancreas Differentiation

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The ability to generate therapeutic cell types from stem cells is at the heart of many envisioned procedures in the promising field of regenerative medicine. Extensive research investigating endoderm development and pancreatic organogenesis has resulted in a comprehensive understanding of key signaling events driving pancreas development, detailed transcriptional profiles of pancreatic progenitor cells, and the developmental trajectories of hormone-producing cells in the endocrine pancreas. Exploiting this knowledge, many labs have shown stepwise guided differentiation of embryonic stem (ES) cells can effectively generate pancreas-like lineages, including insulin-producing beta-like (β) cells. However, these cells have not proven robust in transplantation or functional characterization assays, suggesting that while they express many β-cell markers, they lack critical pancreatic functions. A better understanding of the transcriptional regulatory network guiding cell type specification will help identify how these critical functions are controlled and help improve the directed differentiation of β-cells from stem cells. In these studies, we examine the role of enhanced neurogenin 3 (Ngn3) expression in promoting ES cell differentiation to endocrine pancreas cell types.
The transcription factor Ngn3 is a crucial regulator of endocrine cell specification and is required for promoting pancreatic islet maturation including the development of insulin-producing β-cells and the maintenance of islet function within the mature pancreas. Our research has shown that Ngn3 can direct ES cell differentiation toward the endocrine pancreas lineage, and highlights the importance of timed induction of this protein for proper islet differentiation. Our present data indicates that definitive endoderm populations derived from ES cells are responsive to Ngn3 signaling throughout stepwise directed differentiation protocols but earlier stages of ES cell differentiation are less receptive to Ngn3 activity. Future studies using an inducible transgenic system will provide a tractable experimental platform to study cellular cross-talk between Ngn3-positive and Ngn3-negative cells during pancreas differentiation.

Poster Board #7

The Yb Body, a Major Site for Niche Signaling, mediates piRNA Biogenesis in Somatic Cells

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Despite exciting progress in understanding the piRNA pathway in the germline, less is known about this pathway in somatic cells. We showed previously that Piwi, a key component of the piRNA pathway in Drosophila, is regulated in somatic cells by Yb, a novel protein containing a RNA helicase-like motif and a Tudor-like domain. Yb is specifically expressed in gonadal somatic cells and regulates piwi in somatic niche cells to control germline and somatic stem cell self-renewal. However, the molecular basis of the regulation remains elusive. Here we report that Yb recruits Armitage (Armi), a putative RNA helicase involved in the piRNA pathway, to the Yb body—a cytoplasmic sphere to which Yb is exclusively localized. Moreover, co-immunoprecipitation experiments show that Yb forms a complex with Armi. In Yb mutants, Armi is dispersed throughout the cytoplasm, and Piwi fails to enter the nucleus and is rarely detectable in the cytoplasm. Furthermore, somatic piRNAs are drastically diminished, and soma-expressing transposons are de-silenced. These observations indicate a crucial role of Yb and the Yb body in piRNA biogenesis, possibly by regulating the activity of Armi that controls the entry of Piwi to the nucleus for its function. Finally, we discovered putative endo-siRNAs in the flamenco locus and the Yb-dependence of their expression. These observations further implicate a role for Yb in transposon silencing via both the piRNA and endo-siRNA pathways.

From Embryonic Stem Cell to Epiblast

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A series of pluripotent cell types characterizes early mammalian development: the zygote, the inner cell mass, and finally epiblast. Epiblast is a pluripotent cell type, able to generate ectoderm, mesoderm, and endoderm, but has lost the ability to generate most extraembryonic tissues. We are interested in understanding the molecular events that restrict the pluripotential state and promote epiblast differentiation, and will use embryonic stem cells (ESCs) as an easily manipulated in vitro model. We hypothesize that an FGF5+ epiblast intermediate will be observed during the differentiation of ESCs toward the neural lineage. FGF5 is an established epiblast marker. qRT-PCR and immunocytochemistry analysis of ESCs undergoing neural differentiation suggest a transient FGF5+ epiblast population is present at around 3-4 days. We aim to isolate ESC-derived epiblast and determine its developmental potential. FGF5 cannot be used for this purpose because it is a secreted ligand and very little is known about the regulatory sequences driving expression of FGF5 in epiblast. We are currently screening for genes that display a similar temporal expression pattern to FGF5, with the hope of identifying cell surface markers or genes that can be used to generate a reporter cell line. Preliminary deep sequencing data verifies the expected temporal pattern of FGF5 expression. Once we are able to isolate ESC-derived epiblast, we will begin to investigate the molecular events regulating transition to epiblast. We hypothesize roles for the FGF, Notch, and Hh signaling pathways. A better understanding of this molecular regulation will aid in efficiently generating ESC-derived material for cell-based therapies.

Genome-wide Analysis of mRNA and Protein Targets of PUM1 in Maintaining hESC Pluripotency

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The Pumilio (a.k.a. PUF) family proteins are evolutionarily conserved translational regulators. There are two human Pumilio homologues, PUM1 and PUM2. We have previously demonstrated that the Drosophila PUM protein is essential for stem cell maintenance. However, the role of human Pumilio proteins in maintaining hESC self-renewal remains unexplored. Here, we report that both PUM1 and PUM2 are abundantly expressed in hESCs. By applying RIP-ChIP, and Co-IP-mass spectrometry methods, we have identified more than 500 putative mRNA targets and over 100 putative protein partners of PUM1. These results provide a basis for further analysis of PUM-mediated mechanisms of gene regulation in hESCs.

Tracing the Derivation of Embryonic Stem Cells from the Inner Cell Mass by Single Cell RNA-Seq Analysis

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The molecular mechanism underlying the transition from the inner cell mass (ICM) of blastocysts to pluripotent embryonic stem cells (ESC) is not fully understood. This is partly because of the apparent heterogeneity amongst a small group of cells, which poses difficulties in investigating this question. Using single cell RNA-Seq transcriptome analysis at the resolution of single cells, we have analyzed the dynamic molecular network within individual cells from the ICM outgrowth and the established ESC. This study has identified molecular changes that accompany this transition. Our study shows that key genes that confer the property of self-renewal are up regulated as ICM cells progress to ESC. We also detected very significant global changes of transcript variants from individual genes, amongst which the general metabolism genes are strongly over-represented. Furthermore, there was a global increase in the expression of repressive epigenetic regulators with a concomitant decrease in gene activators. The unique ESC epigenotype may thus be sustained while retaining an inherent plasticity for differentiation. Moreover, changes in miRNAs result in one set that targets early differentiation genes, and the second set targets ESC specific pluripotency genes to maintain a delicate balance between pluripotency and a capacity for rapid differentiation.
In conclusion, our study provides insight into the dynamic and systematic molecular changes that occur during cell fate decisions from identical cells. During the conversion of ICM cells to ESCs, there is an evident arrest of a normal developmental program, which is subverted in vitro in favor of a dynamically stable status for unrestricted self-renewal while retaining the full potential to undergo differentiation into all the diverse cell types. We demonstrate how both the retention of the expression of a full set of master genes allows inheritance of a key property of the ICM, namely pluripotency, while the up regulation and down regulation of other crucial genes permits exit from a normal developmental program, which at the same time confers the key property of unlimited self-renewal. Changes in epigenetic regulators apparently allow for the stable transmission and robust maintenance of the newly acquired epigenotype in ESCs between exceptionally fast cell generations. The conversion is also regulated by miRNAs, with distinct sets of these non-coding RNAs that allow both self-renewal while the cells retain the potential to respond rapidly for to cause differentiation.

PAPI, a Novel TUDOR-Domain Protein, Complexes with AGO3, ME31B and TRAL in the Nuage to Silence Transposition

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The nuage is a germline-specific perinuclear structure that remains functionally elusive. Recently, the nuage in *Drosophila* was shown to contain two of the three PIWI proteins--AUBERGINE and ARGONAUTE3 (AGO3)--that are essential for germline development. The PIWI proteins bind to PIWI-interacting RNAs (piRNAs) and function in epigenetic regulation and transposon control. Here we report a novel nuage component, PAPI (*Pa*rtner of *PI*WIs), that contains a TUDOR domain and interacts with all three PIWI proteins via symmetrically dimethylated arginine residues in their N-terminal domain. In adult ovaries, PAPI is mainly cytoplasmic and enriched in the nuage, where it partially colocalizes with AGO3. The localization of PAPI to the nuage does not require the arginine methyltransferase dPRMT5 or AGO3. However, AGO3 is largely delocalized from the nuage and becomes destabilized in the absence of PAPI or dPRMT5, indicating that PAPI recruits PIWI proteins to the nuage to assemble piRNA pathway components. Expectedly, *papi* deficiency leads to transposon activation, phenocoying piRNA mutants. This further suggests that PAPI is involved in the piRNA pathway for transposon silencing. Moreover, AGO3 and PAPI associate with the P body components TRAL/ME31B complex in the nuage and transposon activation is observed in *tral* mutant ovaries. This suggests a physical and functional interaction in the nuage between the piRNA pathway components and the mRNA-degrading P-body components in transposon silencing. Overall, our study reveals a function of the nuage in safeguarding the germline genome against deleterious retrotransposition via the piRNA pathway.

Transcriptome Analysis of Human Embryonic Stem and Neural Lineage Cells by Deep Sequencing

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Understanding how the dynamics of gene expression influence differentiation is one of the central topics in developmental biology. Animal models have immensely aided our ability to dissect developmental pathways – in particular, the development of the nervous system. Fate-mapping, stage-specific knockouts, and other techniques, performed in vivo on adult and fetal animals, have helped us understand how genes and signaling cascades pattern the journey from stem cell to progenitor to mature neuron. Ethical and moral concerns, of course, preclude our ability to perform these types of experiments in humans.

Human embryonic stem cells (hESC) have the capability to give rise to all the differentiated cell types in adult human tissues and can be driven toward particular lineages; thus, they are an ideal system for studying development. While many studies have characterized the patterns of gene expression in hESC, differentiating, and differentiated cells, most have employed microarrays, which carry the inherent bias of relying on defined sets of oligonucleotide probes and are sub-optimal for analyzing alternative splicing. Ultra-high-throughput ("next-generation") sequencing is a new technology that allows examination of entire transcriptomes with single-nucleotide resolution. It disambiguates the mapping of splicing events and is more sensitive than microarray, allowing detection of low-abundance transcripts. We used deep sequencing to analyze gene and transcript-level expression patterns in a time-series of H9 hES cells as they differentiate down the neural lineage into spinal motor neurons. We have identified numerous transcription and splicing changes that occur during neural differentiation. These results provide new information regarding the differentiation of motor neurons and the gene expression characteristics of hES cells.

The Mili-Setdb1 Complex ss Essential for Pirna-Mediated Transposon Control During Spermatogenesis

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The Piwi family of proteins plays essential roles in germline development, stem cell self-renewal, epigenetic regulation and transposon silencing, and are implicated in oncogenesis. However, the mechanism of epigenetic control during spermatogenesis is largely unknown. The binding of Piwi proteins to Tudor family members via the symmetrical dimethylarginines (sDMA) is evolutionarily conserved and is important for germline development. SetDB1, a histone-lysine methyltransferase containing two Tudor domains, one methyl-CpG binding domain (MBD), one pre-Set domain, and two Set domains, modulates chromatin structure and regulates transcription.

Here we report that SetDB1 is specifically expressed in germ cells and is required for spermatogenesis. Loss of SetDB1 results in the spermatogenic arrest at zygotene stage of meiosis. In setdb1 testis-specific conditional knockout mouse, transposon activity is up-regulated and apoptosis in germ cells is elevated compared to wildtype cells. Furthermore, SetDB1 colocalizes and interacts with Mili, a Piwi homolog in mouse. Both SetDB1 and Mili bind to transposon sequences. Thus, we propose that the Mili/SetDB1 complex is essential for piRNA-mediated transposon silencing during mammalian germline development.

Generation and Characterization of Monoclonal Antibodies against Early Stages of Human Development

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The ability of Human Embryonic Stem Cells (hESCs) to differentiate into all adult human tissues makes them an ideal source of cells for regenerative therapies. However, the direct use of these cells is deleterious due to tumor formation and uncontrolled differentiation. Therefore, hESCs must be differentiated to restricted intermediate progenitors. This should be accomplished in a controlled and reproducible fashion combined with techniques allowing the identification and isolation of such intermediates.
Using coculture systems with stromal cell lines, we have developed a differentiation protocol for hESCs that we hypothesize recapitulates very early stages of human development. Analysis of differential expression of various cell surface markers, using multi color flow cytometry, indicated that these derivatives are heterogeneous and can be dissected into discrete subpopulations. Using anti-CD34 and anti-CD44 antibodies these cultures showed at least 6 discrete subpopulations. Purification of these fractions by fluorescence activated cell sorting and analyses of their gene expression through low-density arrays, together with reactivities against other cell surface markers, have identified populations expressing signatures features of mesenchymal, hematopoietic and vascular lineages. The potential of these populations to progress towards hematopoietic and vascular pathways have been validated in *in vitro* differentiation assays.
We have used these heterogeneous populations of hES derivatives as antigens to generate new monoclonal antibodies using standard hybridoma technology. A battery of over 150 monoclonal antibodies has been generated. Initial characterization of the antibodies has identified molecules able to recognize discrete populations within the 6 populations initially defined by CD44 and CD34. These novel monoclonal antibodies should allow us to further characterize and isolate more defined populations of cells for further dissection of early human developmental pathways to identify tissue specific stem cells for regenerative therapies.
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Lin28 Recognizes Unique Structural Features in its Target Mrnas

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Abundantly expressed in human embryonic stem cells (hESCs), Lin28 is among a handful of key stem cell factors that together can reprogram human somatic cells to a pluripotent state. Mechanistically, Lin28 inhibits the expression of let-7 miRNAs but also exhibits let-7-independent functions. Using a genome-wide approach we have recently shown that Lin28 selectively binds to approximately 5% of mRNAs in hESCs, among which are those for ribosomal proteins and metabolic enzymes (Peng et al., 2011). We further provided evidence that Lin28 stimulates the translation of many of its target mRNAs that are important for the growth and survival of hESCs.

What might be the sequence/structural motifs underlying the specific recognition of target mRNAs by Lin28? Using in vivo reporter gene assays and in vitro gel shift analysis, combined with computer program prediction, we have identified specific structural motifs that are common to all four Lin28 target mRNAs tested. Importantly, using the rules deduced we have been able to predict Lin28-binding sites in other target mRNAs, including those in mice.

Reference

Peng S, Chen L-L, Lei X-X, Yang L, Lin H, Carmichael GG, and Huang Y (2011) Genome-wide studies reveal that Lin28 enhances the translation of genes important for growth and survival of human embryonic stem cells. Stem Cells, doi: 10.1002/stem.591.

Exposure of Fungal Volatile Organic Compounds Causes Toxicity in Human Embryonic Stem Cells

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Microbial growth in damp indoor environment has been correlated with health risk. This study was aimed to determine the cytotoxicity of 1-octen-3-ol, a major fungal volatile organic compound (VOCs) associated with mushroom and mold odors, on human embryonic stem cells (hESCs). 1-octen-3-ol exists as enantiomers, (R)-(-)-1-octen-3-ol and (S)-(+)-1-octen-3-ol, which exhibit identical physical and chemical properties but different three-dimensional orientation. We performed cytotoxic assays by exposing hESCS to racemic 1-octen-3-ol and its enantiomers, (R)-(-)-1-octen-3-ol and (S)-(+)-1-octen-3-ol using an airborne exposure technique. Racemic and (S)-(+)-1-octen-3-ol exhibited greater cytotoxicity to hESCs than (R)-(-)-1-octen-3-ol. The Inhibition concentration 50 (IC50) values for these fungal VOCs were 40-80 fold lower than that of vapor phase toluene, an industrial chemical used as a positive control in this study. Our report pioneers modeling of human embryonic stem cells as an in vitro approach to screen the toxicity of VOCs associated with building-related illness and indicates that in the vapor phase, the fungal natural product 1-octen-3-ol and its enantiomers are more toxic than toluene to hESCs.

Poster Board #17

Probing the Role of Pumilio-Mediated Translational Regulation in Embryonic Stem Cell Self-Renewal and Differentiation

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Embryonic stem (ES) cells are defined by their dual abilities to self-renew and to differentiate into any cell type in the body. This vast potential is precisely controlled by spatial and temporal gene regulation at transcriptional, post-transcriptional, and epigenetic levels. Recent work has identified several transcription factors that are essential for stem cell self-renewal and pluripotency, and current efforts are focused on defining the circuitry and mechanisms of these pathways. The role of translational regulation, however, is virtually unexplored in embryonic stem cell biology. Translational control is a fundamental mechanism of gene regulation during early development in many species, and will likely explain the discrepancies observed between the transcriptome and proteome profiles of stem cells and their differentiated progeny.

Pumilio proteins are well-characterized RNA-binding proteins that act as translational repressors and are reuiqred for germline stem cell maintenance in Drosophila. However, relatively little is known about the two mammalian Pumilio proteins, Pum1 and Pum2. Here we show that Pum1 and Pum2 are both abundant, diffuse cytoplasmic proteins in mouse embryonic stem cells. We have identified over 500 mRNA targets and 3 novel protein binding partners of Pum1 in mouse ES cells via RNA Co-immunoprecipitation Microarray (RIP-ChIP) and mass spectroscopy, respectively. In addition, we have generated Pum1 -/- ES cells and a Pum1 knockout mouse as a means to unravel the function of these proteins in ES cells and during early development. We anticipate that this study will allow us to effectively probe the rule of translational regulation in embryonic stem cell division and differentiation.

The Role of a Novel Piwi-Interacting Protein in Spermatogenesis

Jonathan P. Saxe, Mengjie Chen, Mei Zhong, Hongyu Zhao, Haifan Lin.
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Stem cells play central roles in development and tissue maintenance, with tremendous therapeutic potential. Self-renewing divisions of germline stem cells ensures the maintenance of this cell population throughout the life of the organism. The piwi genes in Drosophila, C. elegans, planaria, and mice have been found to be essential for germline stem cell self-renewal and other gametogenic processes. Piwi proteins bind a novel class of small RNAs, termed piRNAs, which function at least in part to control epigenetic status of chromatin and to suppress transposons; however, it is highly likely that other roles exist. To further understand these potential roles of Piwi proteins, we have identified a novel interactor of Drosophila Piwi, whose mammalian ortholog, Tdrkh, interacts with the mouse Piwi proteins Miwi and Miwi2. Tdrkh contains two KH domains and a Tudor domain, which it utilizes to bind symmetrically dimethylated arginine residues in Miwi and Miwi2. These interactions are fundamentally important to piRNA biogenesis, as Miwi2 is mis-localized in Tdrkh-/- animals with a concomitant global loss of piRNAs. Furthermore, genetic deletion of Tdrkh results in spermatogenic arrest in pre-pachytene spermatocytes, due in part to abnormal DNA demethylation and resulting activation of retrotransposons. Thus, Tdrkh plays a central role in the piRNA pathway, is required for male fertility, and is a crucial component of the maintenance of genetic integrity.

Substantial Mouse Calvarial Bone Defect Healing by Human Embryonic Stem Cells

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Human embryonic stem cells (hESCs) may one day provide an unlimited source of progenitor cells for orthopaedic repair applications. Previous attempts at in vivo bone repair using osteogenic progenitor cells derived from hESCs were complicated and not successful due to a lack of homogeneity of the cells within the cultures, sparse new bone formation, and the formation of teratomas after in vivo implantation. Our objective was to identify a simple, highly efficient method for deriving a large population of osteogenic precursor cells from hESCs and demonstrate successful in vitro and in vivo osteogenesis by these cells. Based on the more narrow potentiality of osteo-chondrogenic progenitors (OCP) derived from hESCs using a simple protocol as described in [1] we evaluated their capability for in vitro and in vivo osteogenesis. While the cells demonstrated a delayed and incomplete ability to differentiate into osteoblasts within in vitro cultures, substantial new bone via endochondral ossification was observed 6 weeks after in vivo implantation in mouse calvarial bone defects. ALU in situ hybridization revealed that human cells were present within the new bone as osteocytes and within a thick cell-rich layer above the new bone, but not in the original mouse bone. Adult mesenchymal stem cells from marrow had limited inconsistent healing. This is the first demonstration of substantial new in vivo bone formation by hESC-derived cells with no evidence of teratoma formation. The high efficiency and simplicity of the process make the method a suitable approach for future attempts at deriving a large population of progenitors for bone tissue engineering from iPSCs.
[1] Boyd NL et al. Tissue Eng Part A. 2009;15:1897-907.

Poster Board #20

Lung Epithelial Cells Arise Predominantly from Non-Hematopoietic Bone Marrow Cells and Rarely from Hematopoietic Cells

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Previous studies have demonstrated that bone marrow (BM) derived cells differentiate into non-hematopoietic cells of multiple tissues. To date, it remains unknown which population(s) of BM cells are responsible for this engraftment. To test the hypothesis that non-hematopoietic stem cells in the BM are the source of marrow derived lung epithelial cells, hematopoietic or non-hematopoietic BM cells were transplanted into irradiated surfactant-protein-C (SPC)-null mice. Donor-derived, SPC-positive type 2 pneumocytes were predominantly detected in the lungs of mice receiving non-hematopoietic cells, but were exceedingly rare in mice receiving hematopoietic cells. We conclude that cells contained in the non-hematopoietic fraction of the BM are the primary source of marrow derived lung epithelial cells suggesting that these may be a primitive stem cell population residing in adult BM.

Poster Board #21

Development of Biomimetic Collagen/Apatite Scaffolds for Bone Repair

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Bone is one of the most implanted tissue. Collagen/apatite composite has been widely investigated and its scaffold has been used in bone tissue engineering. However, precise control of the micro and macro structure of the scaffold to mimic those of natural bone remains a challenge. In the present work, collagen/apatite scaffolds were synthesized by a co-precipitated method using collagen containing m-SBF. Two different pore structures, oriented pore structure and uniform cellular structure, were formed by controlling the lyophilization process. The composition and the morphology of the scaffold were characterized by X-ray diffraction (XRD), thermal gravimetric analysis (TGA) and field emission scanning electron microscope (FESEM), respectively. It was found that controllable mineralization of collagen was achieved by controlling the bicarbonate concentration and flow rate in the m-SBF. Needle-shaped apatite particles with a size of ~100 nm distributed uniformly along the collagen fibers which further densly packed to form an ordered network. Two processing routes for freeze-casting of collagen/apatite slurries were presented. First, a lamellae structure was successfully created by the unidirectional freeze casting and the lamellae had average thickness of 20-200 μm, depending on the freezing temperature and the mineral content. Second, a uniform cellular structure with an average pore size ranging from 50~150 μm was also formed by a constant freeze rate method. The biological performance of the collagen/apatite scaffolds were investigated in vivo using a two-hole mouse calvarial model loaded with osteogenic cells. New bone formation was observed in defect after implantation of scaffolds with different pore size for 4 weeks.

Generation of BAC Fluorescent Protein Reporter Mesc Lines to Study Axial Skeletal Development

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There is limited knowledge regarding how to guide the differentiation of embryonic stem cells (ESCs) into different skeletal lineages, such as osteoblast and chondrocyte. Furthermore, it is not known at what point in the differentiation process will the ideal progenitor cell population(s) exist. With this in mind, we have gone through efforts to generate two catagories of BAC fluorescent protein reporter mouse ESCs lines. The first category focuses on the development of a multi-reporter mESC line to study early embryonic lineage events progressing toward axial skeletal development. For this mESC line, three gene markers were chosen: mesoderm- Brachyury, paraxial mesoderm-Tbx6 and sclerotome-Pax1. Brachyury-mCherry and Pax1-Teal BAC reporter constructs were introduced into a Tbx6-YFP knock-in mouse ES cell line and 6 double positive clones were identified out of 63 total clones. Upon differentiation in the presence of 50ng/ml Wnt3a, Brachyury-mCherry reporter activity was significantly induced, which is consistent with past studies that have showed Brachyury is a direct target of Wnt3a. The second category focuses on mESC lines that report mature skeletal cells types for osteoblasts and chondrocytes. For this, mESC lines were derived from Integrin Binding Sialoprotein-Topaz and Collagen, type II, alpha1 –ECFP reporter mice. Four osteoblast/chondrocyte dual reporter 4 mESC lines were generated. Mouse chimeric studies confirmed multipotency and the ability for these that newly derived cell lines to accurately mark bone and cartilage. Studies on the generation and preliminary characterization of these mESC lines will be presented.

The Role of Piwi Family Proteins in Mammalian Hematopoiesis and Leukemia

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During leukemic cancer progression a rare population of cells, called cancer stem cells, actively contribute to the maintenance of the malignancy. It is possible that normal stem cells and cancer stem cells share common molecular features and identifying key proteins involved in maintaining these cancer stem cells is an area of active investigation. The PIWI gene family encodes proteins that have highly conserved roles in germ-line stem cell maintenance and are known to bind small non-coding RNAs, called PIWI-interacting RNAs (piRNAs). We are investigating a function of the human Piwi homologs and their piRNA partners during normal hematopoiesis and leukemogenesis in order to better understand the mechanism of hematopoietic stem cell self-renewal and its implication in the establishment and maintenance of stem cell driven hematological malignancies. Consistent with previous findings, we observed high expression of the human piwi homolog, piwil4, in undifferentiated blood cell types of normal fractionated human peripheral blood and normal mouse bone marrow. We also observed that piwil4 is highly overexpressed in primary human leukemic samples representing various subtypes of leukemia. Significant up-regulation of piwiL4 is observed in a primary Acute Promyelocytic Leukemia (APL) patient sample and the NB4 leukemic cell line, representing AML subclassification FAB M3. PiwiL4 levels decreased at both the protein and RNA level after differentiation of NB4 cells to mature neutrophils in the presence of retinoic acid (ATRA). Together, these data suggest that both human and mouse piwi genes may play a function in undifferentiated cell types during normal hematopoiesis and overexpression of PIWIL4 may be correlated with maintaining undifferentiated leukemic cell states.

Poster Board #24

Embryonic Stem Cell-Derived Thymic Epithelial Cell Progenitors Enhance T Cell Reconstitution after Bone Marrow Transplantation

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T-lymphocytes (T-cells) play a central role in the adaptive immune system by protecting against infections and neoplasia. T cell deficiencies can occur in a number of physiological and pathological situations. For examples, thymic involution during aging represents the most important cause of thymic atrophy resulting in decreased number and functional activities of T-cells in the elderly. Various genetic and infectious diseases (such as AIDS) are associated with T-cell deficiencies, as is intensive chemotherapy or radiotherapy of cancer. In addition, the recovery of T cells after bone marrow transplantation (BMT) is often slow and incomplete.

T cell development in the thymus depends on the thymic microenvironment, of which thymic epithelial cells (TECs) are the major components. We have demonstrated that embryonic stem cells (ESCs) can be selectively induced in vitro to differentiate into cells that have the phenotype and genotype of thymic epithelial progenitors (TEPs). When placed in vivo, these ESC-derived TEPs self-renew, develop into all types of TECs, and reconstitute the normal thymic architecture. Functionally, these ESC-derived TEPs enhanced T cell regeneration after BMT in animals. In addition to providing a model to study the molecular events underlying TEP and TEC development, the ability to selectively induce the development of TEPs in vitro from ESCs has important implications regarding the prevention and/or treatment of T-cell immunodeficiencies.

Heterotopic ossification is mediated by bipotent BMP-responsive progenitors in the skeletal muscle interstitium

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University of Connecticut

Heterotopic ossification is a debilitating condition that can result from traumatic injury, surgery or genetic disease. We investigated the cellular origins of heterotopic bone in the mouse using Cre/loxP lineage analysis and bioassays of heterotopic ossification based on intramuscular injection of bone morphogenetic protein 2 (BMP2). We demonstrate that osteogenic progenitors that express the receptor tyrosine kinase, Tie2, pre-exist in the skeletal muscle interstitium and are not derived from the endothelium or from hematopoietic lineages. Prospectively isolated Tie2+ progenitors, which were purified to apparent homogeneity by FACS, robustly contributed to BMP2-dependent heterotopic ossification after cell transplantation. Clonal analysis of FACS-fractionated cells revealed that these progenitors are bipotent, exhibiting the capacity for both spontaneous adipogenic differentiation and BMP-dependent osteogenic differentiation. Identifying the cells-of-origin responsible for heterotopic ossification provides a potential therapeutic target to treat, mitigate or prevent this disabling condition.

Methods for Generating Monocyte Progenitors from Human Pluripotent Stem Cells

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The development of protocols allowing hematopoietic progression from hESCs is important as hematopoietic progenitors can be used for therapies and to study early blood development.

We have identified monocyte progenitors able to generate macrophages, dendritic cells and osteoclasts. We hypothesize that these progenitors could be transplantable contributing to multiple physiological processes. We have focused on the development of osteoclasts, which are specialized cells that develop as terminally differentiated bone resorbing cells in the context of bone endosteum. We propose that this feature makes them optimal vectors to deliver bioactive molecules to modify bone marrow microenvironments. This could impact areas such as bone marrow transplantation, tumor bone metastasis and bone fracture repair. We could accomplish this by developing monocyte progenitors from genetically modified human pluripotent stem cells expressing the gene(s) of interest.

We have implemented two methods for forming functional osteoclasts from hESCs. The first is based on cocultures between hESCs and mouse stroma cells and the second on the development of embryoid bodies under hematopoietic inducing conditions. These methods can reproducibly generate derivatives with hematopoietic characteristics with different efficiencies. These early hematopoietic derivatives can be isolated by flow cytometry, expanded in early hematopoietic cytokines and further cultured in osteoclastogenic conditions generating osteoclasts identified as TRAP positive multinucleated cells with the ability to resorb mineral matrix when developed over bone slices.

Using antibodies against cell surface markers expressed in early monocytic lineage, we have been able to identify intermediate stages that could represent osteoclast-committed progenitors. These include cells with the phenotypes CD45^{+} CD34^{+} CD38^{-} CD45RA^{+} CD115^{+}, and CD45^{+} CD14^{+} CD115^{+} CD117^{+}. We are currently evaluating the individual potential of these populations to generate osteoclasts *in vitro*. We plan to perform clonal studies and to evaluate the ability of these progenitors to form osteoclasts *in vivo* upon transfer into immunodeficient animal models.
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Increased Kinetics and Efficiency of Human Somatic Cell Reprogramming Using Small Molecules

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Reprogramming human somatic cells remains a highly inefficient and time-consuming process. Small molecules targeting specific signaling pathways have been shown to enhance reprogramming and/or replace the transcription factors required for reprogramming. In this study, chemical compounds were screened for their effects on enhancement of reprogramming efficiency as assessed by the number and the overall quality and morphology of human iPS colonies generated. A small molecule boost cocktail was identified that increased the number of iPS colonies by 2-3 fold. The colonies formed possessed the distinctive flat 2D morphology that is reminiscent of human embryonic stem cells and could be easily passaged in contrast to the untreated control that exhibited 3D morphology and were difficult to passage. Furthermore, the time to establishment of full reprogrammed human iPS colonies that were SSEA4+, TRA-1-60+, Hoechst-Dim was significantly reduced from 50-60 days to 30 days. Reprogramming with small molecules was further validated on multiple human fibroblast cell lines in both feeder and serum-free, feeder free culture systems. In summary, the use of the polycistronic lentiviral expression cassette in combination with small molecules provide a convenient platform for screening small molecules that enhance reprogramming efficiency with the eventual aim for generating iPS cells without genetic modification to the genome.

Evaluation of Klf4 Recombinant Proteins for Improved Reprogramming

Yong Tang, Chih-Jen Lin, X. Cindy Tian.
University of Connecticut

Induced Pluripotent Stem Cells (iPSCs) are Embryonic Stem Cell (ESC) - like cells which are reprogrammed directly from somatic cells, by ectopic expression of the reprogramming factors (RFs), including Oct4, Sox2, Klf4, and c-Myc. iPSC technology hold great potential in stem cell therapy by generating patient-specific pluripotent cells and by circumventing the ethical issue of ESC derivation from human embryos. However, the introduction of RFs to cells by viral or other vectors raised concerns over tumorigenicity due to genomic DNA integration. iPSCs could be induced by reprogramming proteins (piPSC) linked to cell penetrating peptides (CPPs) such as poly-arginines. This approach bypasses genomic alteration to the cells. However the piPSC induction efficiency was extremely low and the optimal protein transduction conditions were not well established. We aimed to evaluate the reprogramming potency of recombinant proteins fused to the CPPs of human immunodeficiency virus transactivator of transcription (HIV TAT). We expressed and purified various recombinant Klf4, conducted in vitro protein functionality tests, and compared their reprogramming potency using a combined protein/retroviral Oct4, Sox2, and c-Myc induction assay. We found that TAT linked Klf4 proteins can effectively induce iPSC colony formation in 2 to 4 weeks from fibroblasts. We further evaluated key parameters in piPSC induction, including protein transduction dosage, frequency, and transduction cycles. We found that a 4-cycle-transduction of recombinant Klf4 is sufficient for effective iPSC induction. Our study provided a valuable strategy to evaluate the potency and transduction conditions for recombinant RFs in order to improve the piPSC induction efficiency.

Poster Board #28

Studies of Type I Collagen Mutations in Type I and IV Osteogenesis Imperfecta (OI) Patients Induced Pluripotent Stem (iPS) Cells

Xiaonan Xin, Xi Jiang, Liping Wang, Mary Louise Stover, Hui-Chen Chan, Yongxing Liu, Liisa Kuhn, David Rowe, Alexander Lichtler.
Reconstructive Science, University of Connecticut Health Center

Osteogenesis imperfecta (OI) is a heritable bone disease caused by mutations in the type I collagen genes. Severe forms have defective collagen fibers with inherently unstable structures causing bone fragility. Previously we reported initial derivation of iPS cells from patients with severe and mild OI, with the long term goals of investigating OI pathogenic mechanisms, and testing therapeutic strategies. In the current studies we have further characterized the cells we produced, and we are in the process of investigating their mutant collagen proteins. The mutations in the two cell lines have been identified; as expected, the Type I OI cells contain a premature termination codon that leads to mRNA instability, and the Type III/IV cells have an alanine substituted for the glycine at amino acid position 491. RNA analysis using an ABI pluripotency low density array PCR plate showed a similar gene expression profile of the iPS cells to H9 hES cells. With the goal of identifying defined, non-human factor-free culture conditions for the iPS cells, we have shown that our cells have similar gene expression profiles when cultured in Stemgent NutriStem media compared to standard mouse embryo fibroblast conditioned media. We have developed a method for differentiating our iPS cells into a mesenchymal stem cell-like population that is over 95% CD73+ and CD44+, and negative for hematopoietic cell markers. Low density array PCR analysis demonstrated expression of mesodermal markers in the differentiated cells, and Type I collagen mRNA is also expressed. We assessed the ability of the iPS cells to produce teratomas that contain bone. We are working to develop a method for differentiating hES cells and iPS cells derived MSC into osteoblasts in our calvarial defect model with NOD-*scid IL2rg null* background mice, and we are using adult human bone marrow derived MSC in this defect model to develop analysis methods to evaluate the participation of human cell in new bone formation in the defect region.

FOX Splicing Factors and UBE3A Neuron-Specific Imprint in Angelman Syndrome Ips Cells and Derivatives

Pin-Fang Chen, Marc Lalande, Stormy J. Chamberlain.
University of Connecticut Health Center

Scientists have successfully reprogrammed human somatic cells to embryonic stem cell-like induced pluripotent stem cells (iPSCs), which can be differentiated into virtually any tissue in the human body. The ability to create iPSCs and their derivatives gives access to a variety of live human cells and provides tools for understanding genetic disease mechanisms. We have generated an in vitro model of Angelman syndrome (AS), a neurogenetic disorder. AS is caused by loss of function of the imprinted gene UBE3A. In iPSCs, UBE3A is expressed from both parental alleles. The paternal allele of UBE3A becomes silenced during the process of neural differentiation, leading to the expression of UBE3A exclusively from the maternal allele in mature iPSC-derived neurons. This silencing occurs concomitantly with the paternal expression of RNA transcripts downstream of the SNURF/SNRPN gene, including an antisense transcript of UBE3A (UBE3A-ATS). We hypothesize that splicing changes that occur during neural differentiation result in neuron-specific processing of the downstream transcripts, including UBE3A-ATS, and that the neuron-specific processing of these transcripts leads to the silencing of paternal UBE3A. Using RNA immunoprecipitation with antibodies against the FOX-1 family of splicing factors, we observed changes in the abundance of FOX paralogue binding in chromosome 15q11-q13 as well as in other FOX-binding clusters. Our results suggest that FOX factors may contribute to establishing the neuron-specific UBE3A imprint and that each FOX paralogue may have a unique function during neural differentiation.

Application of Human Pluripotent Stem Cells in Cancer Immunotherapy

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Human pluripotent stem cells represent a unique reservoir that could be utilized for generating cells of a given lineage. Evidently, cancer immunotherapy field could benefit from this, since a key limitation in developing an effective immune based cancer therapy is the lack of sufficient anti-tumor immune effectors in a great majority of cancer patients. The main reason for this is the fact that most human tumor antigens are "self-antigens", and most self reactive anti-tumor immune effectors are deleted during development to avoid autoimmunity. We are developing methods to utilize human pluripotent stem cells in cancer immunotherapy of melanoma. We here present data on our progress towards generating hematopoietic stem cells (HSC) from Human Pluripotent Stem Cells (hPS: hES & iPS) cells, and their characterization.

Poster Board #31

Tracking the Genomic Stability of Human iPS Cells by Both Karyotyping and Comparative Genomic Hybridization

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Genomic stability of human induced pluripotent stem cells (hiPSCs) is essential to safeguard their value for research and therapeutic applications. Here we monitored the genomic stability of 32 hiPSC lines by G-banded karyotyping and high-resolution array-based comparative genomic hybridization (aCGH) analysis. Few aneuploidies were detected in hiPSCs by karyotyping, following extended periods in culture. Using aCGH, we identified unique copy number variation (CNV) signatures for hiPSC lines derived from specific sources of parental fibroblasts, including CNVs shared between high and low passages and CNVs acquired specifically by hiPSCs during culture. Recurrent CNVs at 1q31.3 and 17q21.1 were shared by > 25% of hiPSCs. Furthermore, the loss of 8q24.3 was unique to hiPSCs. Additional recurring CNVs at 2p11.2 and 20q11.21 were acquired by both hESCs and hiPSCs. Our results suggest that both karyotyping and aCGH are required for the analysis of the genomic stability of hiPSC lines.

Generation and Characterization Of iPS Cells from CMD Patients and Healthy Controls

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University of Connecticut Health Center

Studying rare genetic bone disorders is clinically significant due to the lifetime debilitating impact they have on patients and limited treatment options. Obstacles for research in this field include unavailability of tissue specimens and lack of animal models. Rapid advances in induced pluripotent stem (iPS) cell biology opened new avenues to study bone cells from such patients. Previously, we have created a knock-in mouse model carrying a Phe377del mutation in ANK and identified osteoclast involvement in craniometaphyseal dysplasia (CMD), a disease characterized by progressive thickening of craniofacial bones and widening of metaphyses in long bones. We propose to use patient-specific iPS cells to further investigate OC defects in CMD. We began to generate and characterize iPS cells from human skin fibroblasts and from SHEDs (stem cells from human exfoliated deciduous teeth) of CMD patients and healthy controls. Reprogramming of somatic cells into iPS cells was performed by retroviral transduction of Yamanaka's factors (OCT3/4, SOX2, KLF4 and c-MYC) plus Lin28, and by a single lentiviral "stem cell cassette" (STEMCCA) encoding OCT4, KLF4, SOX2 and cMYC. Reprogrammed cells had an indistinguishable morphology from human embryonic stem (hES) cells. These iPS cells expressed SSEA-4, TRA-1-60, TRA-1-81, Oct4, Sox2 and Nanog, as shown by gene expression analysis and immunocytochemistry and formed embryoid body (EB) in vitro. Assays for testing in vivo differentiation capability of these iPSCs by teratoma formation are in progress. To derive OC-like cells from iPSCs, we first applied the published method on H1/H9 cells. These generated multinucleated cells were TRAP positive with actin belts at the periphery and were capable of resorbing calcium-phosphate coated discs suggesting that hESCs, likely iPSCs, can be differentiated to functional OCs. We continuously optimize methods of generating iPS cells and study CMD mouse model to generate hypotheses for subsequent iPS-based human studies.

Differentiating Human Embryonic Stem Cells towards the Auditory Sensory Neuron Precursor

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To treat deafness and hearing loss, cochlear implants bypass damaged inner ear receptor hair cells to directly stimulate the spiral ganglion neurons. To complement that approach in patients with neuron degeneration, we are trying to generate the human otic progenitor, which with further induction and in vivo differentiation can form healthy ganglion neurons. Deriving protocols to differentiate human pluripotent stem cells into various cell types of the human body is the first step towards cell-based therapy. Currently, however, there is no known protocol for generating the human placodal ectoderm and the subsequent otic progenitor. We attempt this by adapting protocols relevant for neural induction to follow expression profile of genes critical for the formation of the ectoderm derivatives and the placodal ectoderm. We find that human embryonic stem cells can differentiate from embryoid body or monolayer adherent culture towards the competent ectoderm expressing a combination of ZIC1, DLX3 and GATA3. This is accompanied or shortly followed by an upregulation of the definitive placode markers including EYA1 and SIX1. Although FGF signal has been shown to be essential for otic placode formation in other model embryo studies, we found that addition of FGF factor at least during the early phase of the human embryonic stem cell differentiation does not support development of the placodal ectoderm. Ongoing studies based on this finding aim to optimize the method of differentiation by locating the stage and time frame to manipulate signaling pathways involving BMP, FGF and WNT, which are known to be important for acquiring the otic fate, in the hope of generating a robust population of otic progenitor which is suitable for translational studies.

Vascular endothelial growth factor receptor 3 directly regulates murine neurogenesis

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Neural stem cells (NSCs) are slowly dividing astrocytes that are intimately associated with capillary endothelial cells in the subventricular zone (SVZ) of the brain. Functionally, members of the vascular endothelial growth factor (VEGF) family can stimulate neurogenesis as well as angiogenesis, but it has been unclear whether they act directly via VEGF receptors expressed by neural cells, or indirectly via the release of growth factors from angiogenic capillaries. Here, we show that VEGFR-3, a receptor required for lymphangiogenesis, is expressed by NSCs and is directly implicated in the control of neurogenesis. *Vegfr3::YFP* reporter mice show VEGFR-3 expression in multipotent NSCs which are capable of self-renewal and are directly activated by VEGFR-3 ligand VEGF-C, *in vitro*. Overexpression of VEGF-C stimulates VEGFR-3-expressing NSCs and neurogenesis in the SVZ, without angiogenic effect. Conversely, targeted deletions of *Vegfr-3* in neural cells and subventricular astrocytes, as well as blocking VEGFR-3 signaling with antibodies reduce SVZ neurogenesis. Therefore, VEGF-C/VEGFR-3 signaling directly regulates NSCs and promotes adult neurogenesis, opening potential approaches for neurodegenerative disease.

Poster Board #37

Doublecortin Expression Promotes the Migration Of hESC-Derived Neurons and Stimulates the Construction of Neural Progenitor Scaffolds that Guide Migration

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Human embryonic stem cell derived neuronal progenitor cells (hNPCs) potentially provide a valuable cell source for cellular replacement following neurodegenerative diseases. Upon appropriate placement in damaged areas, hNPCs should be able to migrate, differentiate, and integrate in the host environment.

One of the greatest challenges in cell replacement therapies is the ability of neuronal progenitor cell populations to migrate into and integrate within existing neuronal circuitry. We hypothesized that migration of hNPCs could be promoted by genetically manipulating them to express genes that promote migration during normal development. The DCX gene encodes doublecortin, a microtubule-associated protein expressed in migrating neuronal precursors which stabilizes microtubules and promotes cell migration. Our present study focused on the effect of human Dcx (hDcx) expression on hESC-dNPC using vitro migration models. For stable transgenesis of hNPCs we used a piggyBac transposon system, which allows for viral-free insertion of genes. We constructed DNA plasmid vectors 5'TRCAGGS- hDcx-IRES-GFP'TR (Dcx-PB) and 5'TR-CAGGS-GFP'TR (GFP-PB) by cloning the full-length human Dcx cDNA or GFP cDNA into the 5'TR-CAGGS-3'TR vector. hNPC were derived from the H9 cell line (UCHC stem cell Cre). Neural rosettes were trypsinized and nucleofected either with (hDCx) or GFP. Similarly sized neurospheres generated from genetically modified rosettes were plated either on Matrigel or rat brain slices prepared from rat neocortex. Migration of cells out of the neurospheres was assessed after 12h, 24h, 48h and 120h. In both assays, hESC-dNP transfected with hDCX migrated significantly further than control cells (GFP transfected). When overlayed on the top of cortical rat slices, hNPC transfected with hDCx migrated 340.97±17.24 µm vs. 153.45±14.6 µm for GFP-transfected neurospheres. Additionally, a network of nestin fibers was found to develop and integrate into host tissue from neurospheres that were transfected with hDCx. DCX expressing neural progenitors that migrated into tissue were invariably found associated with the infiltrating nestin expressing cellular fibers suggesting that enhanced migration into neural tissue following DCX expression is mediated by induced growth of migration scaffolds from neural progenitors. In summary, our results show that hDcx expression significantly improves migration of hNPs. Future experiments are in progress to test whether in vivo transplanted cells migrate more into tissue when transected with hDcx.

Maturation of the Retinal Pigment Epithelium: Effects of Co-Culture with Retinal Progenitors

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Age-related macular degeneration is the leading cause of blindness in patients over 60 in the world. Human embryonic stem cells (hESC) may serve as unlimited donor source of retinal pigment epithelium (RPE) cells for transplantation. This report characterizes the tight junctions of RPE derived from hESC. Embryoid bodies (EBs) were differentiated into RPE-like cells.

Monolayers of pigmented epithelial cells were isolated and cultured on laminin-coated Transwell filters for 6-8weeks. After this time, cultures could be adapted to a serum-free medium that was developed for human fetal RPE. For co-culture experiments, hESC-derived retinal progenitors were cultured on Transwell filters, adapted to serum-free medium and cocultured with hfRPE for 5 weeks. The transepithelial electrical resistance (TER) was used to assess the function of tight junctions. Gene expression of tight junction and RPE maker genes was examined by quantitative real-time RT-PCR, and protein expression was examined by immunoblotting and confocal, immunofluorescence microscopy.

The hESC-derived RPE cells exhibit the polygonal monolayer morphology with melanin granules and RPE-specific gene markers including RPE65. The TER was ~250$\Omega \times cm^2$. Like native fetal RPE, Claudin-19 mRNA was the most prominent mRNA. Minor RPE claudins (-1, -2, -3) were detected in less amounts. Non-native hfRPE claudins (-4, -6, -11 and-14) were also detected. Claudin-19 was evident by immunoblotting and localized to tight junctions. Claudin-3 localized to the tight junction, but also the rest of the lateral membrane. In coculture experiments with hfRPE, retinal progenitors increased the expression of RPE65 and decreased the expression of claudin-1. Conversely, hfRPE increase the expression of rhodopsin by the retinal progenitor cells.

In summary, the hESC-derived RPE cells expressed appropriate RPE markers, including claudin-19 as the predominant claudin, but non-native claudins were also evident. Retinal progenitors may be able to promote maturation of the hESC-derived RPE, as they were able to promote maturation of hfRPE.

Neural Progenitors from Human Embryonic Stem Cells Release a Factor that Increases Synaptophysin Expression

Saranya Santhosh Kumar, Radmila Filipovic, Joseph Loturco.
University of Connecticut

Synaptic degeneration is an important contributing factor for several neurodegenerative disorders. For example, decreased numbers of synapses correlates well with the severity of dementia in Alzheimer's Disease (DeKosky and Scheff,1990), and Frontotemporal Dementia (Clare et al., 2010). Discovery of diffusible factors that prevent deterioration of synapses may provide insights for the design of novel therapeutic approaches, and neurons derived from human embryonic stem cells may be a potential source for such factors. In our study, we tested neuronal progenitor cells derived from human embryonic stem cells for the ability to increase the expression of a presynaptic protein, synaptophysin. We used co-cultures of organotypic brain slices and neural progenitors derived from human embryonic stem cells. We found that human neural progenitors have a stimulatory effect on the expression levels of synaptophysin protein in brain tissue with hESC-dNPs increasing the level of synaptophysin approximately 3 fold above controls. We next tested the hypothesis that this increase in synaptic protein might be due to a factor secreted by human neural progenitors. To test this hypothesis; we performed co-culture experiments and conditioned media experiments. We found evidence for a diffusible factor produced by neurons derived from human embryonic stem cells. Neural progenitors grown in culture with brain tissue but separated from tissue enhanced the levels of synaptophysin in brain slices approximately 3 fold above controls, and similarly conditioned media from neural progenitors added to brain slices increased the levels of synaptophysin in cultured brain slices. Further studies are now in progress to characterize this diffusible substance produced by human progenitor cells.

Morphogenesis, Patterning, and Differentiation of ESC-Derived Neural Rosettes

Erin Banda, Noélle Germain, Laura Grabel.
Wesleyan University

Embryonic stem cell (ESC) in vitro neurogenesis resembles the development of the central nervous system in the early mammalian embryo. Using a well-defined monolayer protocol that utilizes the BMP-antagonist Noggin, we observe formation of neural rosettes, in which differentiating neural stem cells (NSCs) are radially arranged around a lumen and exhibit interkinetic nuclear migration. N-cadherin and Numb are localized at the apical luminal surface, suggesting the emergence of a polarized epithelial layer, reminiscent of radial glia in the embryonic neural tube. Rosettes also appear to have regional identities, for example expressing the ventral marker Nkx2.1 in one half of the rosette. In addition, our RT-PCR analysis shows that during differentiation, cells express markers of both dorsal (Pax7) and ventral neural populations (Mash1, Nkx2.1 and Dlx2). One goal of our work is to use this in vitro system to generate an enriched population of GABAergic interneurons that could be used to treat neurodegenerative diseases like Temporal Lobe Epilepsy (TLE), characterized by the loss of inhibitory GABAergic interneurons. We now demonstrate that further differentiation of hESC-NSCs efficiently produces mature GABAergic neurons.

Dopamine Potentiates Differentiation of Human Embryonic Stem Cells into Neurons

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Department of Neuroscience

University of Connecticut Health Center

Dopamine (DA)-releasing fibers project into many brain structures including the human cerebral cortex. In adult human, the activation of DA fibers plays an essential role in cognitive/behavioral processes. It is not clear why DA-releasing fibers arrive into the cerebral cortex many months prior to establishment of any cognitive faculties, nor why it is important to have DA release in human fetal and neonatal brains before the arrival of thalamic projections and formation of functional circuits. We hypothesize that during the early phase of brain development, characterized by massive neuron proliferation and differentiation, DA serves a trophic factor, rather than a cognitive neurotransmitter. To test if DA present in the developing brain can influence neuronal differentiation, human embryonic stem cells were differentiated in the presence of dopamine using two different methods: [1] a four stage protocol with defined media constituents; or [2] by PA6 stromal cell co-culture. Dopamine exposure during the last two stages of the defined differentiation protocol resulted in a modest increase (173% of control) of dopaminergic neuronal cell clusters. Activation of the cAMP dependent signal transduction pathway by dbcAMP also potentiated the numbers of dopaminergic clusters. The increase caused by DA did not occur in the presence of dbcAMP, suggesting that DA acts through the cAMP/CREB or PKA pathway to produce this effect. L741, an agonist of DR2, which can inhibit cAMP production through G_i, reduced the numbers of TH+ clusters, consistent with the involvement of cAMP in the differentiation process. Early DA exposure, during the embryoid body and primitive neuroepithelial stages, resulted in 2.8 fold more neuroepithelial colonies midway through the differentiation protocol. By the end of the differentiation protocol, early DA exposure resulted in 3.2 fold more neuronal clusters, and thus more TH+ clusters. DA exposure of stem cells differentiating by the second method, PA6 mouse stromal cell co-culture, resulted in increased (172% of control) expression of TH, but no change in numbers of TH+ cell clusters. These results are consistent with a role for DA in directing stem cells towards a neuronal fate, potentiating the differentiation of either committed or uncommitted neurons into dopaminergic neurons, and increasing the expression of TH in neurons.

Interactions between Mouse Embryonic Stem Cell-Derived Neural Progenitors and the Host Brain

Chelsea Lassiter[1], Sandy Becker[1], Sean Mcgill[2] Nate Hartman[3], Laura Grabel[1]

[1]*Wesleyan University*, [2]*Hampshire College*, [3]*Yale University*

Embryonic stem cell (ESC)-derived transplants hold great promise as cell replacement therapies for neurodegenerative diseases. However, it is important to first understand the host brain conditions that will lead to a successful transplant. We use a lesion model, transplanting mouse or human ESC-derived neural progenitors (ESNPs) into the hippocampi of mice that have been given a kainic acid-induced seizure. Within a week after transplantation, the ESNPs have migrated posteriorly along the upper blade of the dentate gyrus (DG), replacing the endogenous cells of the upper blade that have degenerated due to the fluid injection. After four weeks the transplanted ESNPs have integrated into the upper blade, and express the granule cell marker Prox1. Our preliminary data suggest that the transplant recruits host endothelial cells from the surrounding hippocampus and that they form vessels within a week. The number and size of blood vessels is increased in and around the DG on the side of the brain receiving a transplant versus the contralateral control side. In addition we observe that migrating ESNPs are found in close proximity to endogenous blood vessels. Our previous data suggest that the posterior migration of the transplanted cells occurs in response to the chemokine CXCL12 (SDF-1α), and we now show that this chemokine is present on the host vasculature. Since the vasculature is coated with laminin, we are investigating the role of the integrin receptor $\alpha 6\beta 1$. The data reported here suggest a role for blood vessels in supporting the transplant and in providing a migratory scaffold.

The Specification of Nociceptive Neurons from Human Embryonic Stem Cells

Erin Boisvert[1], Shawn E. Hallowell[2], Sandra J. Engle[2], Xue-Jun Li[1]
[1]*University of Connecticut Health Center*, [2]*Pfizer Inc.*

How to direct the differentiation of human embryonic stem cells (hESCs) into functional nociceptor neurons, which are responsible for translating the sensation of pain to the nervous system, remains to be elucidated. Based on our successful establishment of a chemically defined system to generate neural lineage cells and spinal progenitors from hESCs, we aim to investigate the specification and maturation of nociceptor neurons from hESCs by establishing a chemically defined system. Human ESCs were first differentiated into the neural lineage using our system as previously described. The addition of retinoic acid and bone morphogen factor 4 (BMP4) at specific time points and concentrations yielded a sufficient population of neural crest progenitor cells (AP2a+, P75+). These progenitors were then dissociated and plated on polyornithine/laminin coated coverslips for terminal differentiation in the presence of neurotrophic factors (nerve growth factor, glial cell line-derived neurotrophic factor, growth differentiation factor 7). Several weeks after differentiation, nociceptive neurons (TrkA+) were generated in the cultures. These mature neurons also stained positive for sodium channel markers (Nav 1.7) as well as P2X3 which plays a role in the peripheral response to pain. Thus this study provides insights as to some of the important factors which may play a significant role in our ability to yield nociceptive neurons from human embryonic stem cells. The function of these nociceptor neurons and the mechanisms underlying the generation of these neurons are under further investigation.

Deciphering Early Mammalian Neural Crest Development

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Molecular, Cellular and Developmental Biology, Yale University

Neural crest cells contribute to a variety of tissues found throughout the body, including the peripheral nervous system, skin melanocytes, the bones and cartilage in the head, and portions of the cardiovascular system. This astonishing capacity of neural crest cells to generate a broad range of derivatives is unfortunately matched by their excessive participation in human disease. Defects in the development of neural crest cells are associated with cleft/lip palate, aggressive cancers such as melanoma (skin) and neuroblastoma (brain), myelination disorders and Hirschprung's disease. Despite their great impact on human health, little is known about neural crest cells, as they are only briefly present during early human development. This makes them extremely difficult to obtain and study. In fact, human neural crest cells have rarely been isolated and studied in detail at the cellular and molecular levels.

Previously, our lab discovered that neural crest cells arise much earlier in development than initially thought. We identified Pax7 in the chick as a unique marker of early neural crest cells that is required for neural crest development. In mammals, Pax7 is best known as a marker of muscle stem cells, but its role in neural crest development remains largely uncharacterized.

Here, we use genetic, molecular and cellular techniques to better understand mammalian neural crest development and the involvement of Pax7 therein. We show that early human and mouse neural crest cells express Pax7, and that Pax7 descendants in transgenic mice contribute to multiple neural crest tissues. Additionally, we have defined conditions to rapidly enrich for Pax7+ hESC-derived presumptive neural crest precursors that co-express other neural crest markers. When differentiated in bulk, these precursors produce multiple neural crest cell types. These results provide the foundation necessary to develop diagnostic and therapeutic tools based on human neural crest stem cells.

Origin of Dopamine Signaling in the Subventricular Zone

Jessica Lennington, Anna Goodheart, Joanne Conover.
University of Connecticut

The subventricular zone (SVZ) in the adult brain contains stem cells that produce new olfactory bulb neurons throughout life. The factors that regulate this process and the implications of continual neurogenesis in the olfactory bulb are not fully understood. One candidate regulatory molecule is dopamine which is released in the SVZ by mesencephaic dopamine neurons residing between the substantia nigra pars compacta and the ventral tegmental area. Activation of the substantia nigra pars compacta is typically associated with sensorimotor function, while ventral tegmental area is typically associated with motivational learning and modulation of affect. We have characterized the dopaminergic neurons that innervate the SVZ by retrograde tracing studies together with dopamine neuron-specific biomarkers. We found that the SVZ is regulated by and responsive to dopamine signaling from midbrain cells more commonly associated with the ventral tegmental area. These results suggest that midbrain dopamine signaling may support olfactory bulb learning throughout life via regulation of SVZ neurogenesis.

Disease-Modifying Effects of Fetal GABAergic Progenitors Transplanted into the Dentate Gyrus of the Hippocampus in Mice with Temporal Lobe Epilepsy and Spontaneous Recurrent Seizures

Elizabeth Litvina, Stephanie Tagliatela, Xu Maisano, Elizabeth Evans, Lauren Seo, Nicholas Woods, Janice R. Naegele.
Wesleyan University

Alterations in neuronal excitability are a common cause of seizure disorders. In adult temporal lobe epilepsy (TLE) decreased synaptic inhibition in the dentate gyrus of the hippocampus is an important factor in the development of spontaneous recurrent seizures (SRS). As neurodegenerative changes are often associated with severe, drug-resistant TLE, we investigated in mice with TLE, whether transplanted inhibitory GABAergic interneuron progenitors integrate into regions undergoing degeneration and reduce the occurrence of spontaneous seizures. We transplanted approximately 100,000-200,000 neural progenitors into the dentate gyrus or the entorhinal cortex. The neural progenitors were harvested on embryonic day 13.5, from the medial ganglionic eminence (MGE), the primary ventricular zone region for forebrain interneuron neurogenesis. Transplanted cells expressed EGFP or RFP to allow tracking and quantification of graft survival and volume. Spontaneous seizures were monitored in the mice using continuous video-electroencephalographic recordings (v-EEG) for 100 days. Suppression of SRS over the course of the recording period was observed in mice receiving dentate gyrus hilar MGE grafts, but not in the mice with entorhinal grafts. Statistically significant suppression of seizures was typically observed by 40 days post-transplantation and these differences lasted up to100 days of continuous v-EEG monitoring. Approximately 80% of the transplanted cells differentiated into GABAergic interneurons; 25% co-expressed the neuropeptide somatostatin, and additional subsets expressed the calcium binding proteins parvalbumin, calbindin, or calretinin. Dentate but not entorhinal transplants suppressed seizures.
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Characterization of Aging Neural Stem Cells in the Subventricular Zone

Brett Shook, David Manz, Anitha Saravanakumar, Olayinka Edwards, Joanne Conover.
University of Connecticut

In the adult mouse forebrain persistent neurogenesis in the subventricular zone (SVZ) provides a constant stream of new interneurons to the olfactory bulb. With increasing age (20 to 24-months old), the SVZ shows an age-dependant loss of its proliferative capacity, accompanied by a decline in the number of neural stem cells (NSCs). Variability in cell labeling techniques has prevented an accurate assessment of age-related changes in the NSC pool, as molecular markers of NSCs also label non-stem niche cells. NSCs are a subpopulation of SVZ astrocytes that contain an apical process that contacts the lateral ventricle and posess a primary cilium. Ventricle-contacting astrocytes are anchored by adherens junctions to surrounding ependymal cells forming regenerative units at the ventricular surface. By combining whole mount tissue preparation of the lateral ventricle wall with immunofluorscence to identify astrocytic processes contacting the ventricle, accurate identification of NSCs is dramatically improved. Using this strategy, we characterized age-related changes in the stem cell pool and the regenerative SVZ niche over an aging continuum of 3, 6, 12 and 20 - 24 months. Our studies define the time-line of age-related decline in the SVZ and use of markers for different stages of the cell cycle (PH3 and MCM2) allowed us to identify the fraction of NSCs within each stage of the cell cycle. Our analysis reveals a continual decline in the number of regenerative units and the number of NSCs within each unit throughout aging. However, it is important to note that the remaining regenerative units in the aged SVZ are neurogenic.

Efficient Induction, Selection and Differentiation of Pure Populations of Neural Cells from Human Pluripotent Stem Cells Using Aggrewell[tm] and Novel Neural Induction Systems

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Induction of neuroectoderm from human pluripotent stem cells (hPSCs) is the first step in differentiation protocols used to produce neural progenitor cells and more specialized cell types of the CNS such as neurons, astrocytes and oligodendrocytes. However current neural induction protocols and media are not standardized within and between labs, therefore reproducibility of the protocols and results is an on-going problem for researchers. In many neural induction protocols, the formation of embryoid bodies (EBs) from undifferentiated hPSCs is the first step employed to direct these cells towards a neural fate. However the routinely used “scraped EB” or “spin EB” protocols result in non-uniformly shaped EBs of varying sizes which can lead to inconsistent results in neural differentiation. In an attempt to standardize protocols for neural induction from hPSC, we used the AggreWell™ system to generate EBs of defined sizes and a novel neural Induction Medium. EBs that contained 10000 cells per EB were formed in Neural Induction Medium using the AggreWell™800 system. Media changes were performed within the AggreWell™ plate every day. On day 5, EBs were harvested from the AggreWell™ plate and then sub-cultured for an additional 7 days on poly-L-ornithine/laminin coated plates in Neural Induction Medium to generate “neural rosettes”, which is a commonly accepted morphological criterion indicative of early neural progenitor induction. Next, neural rosette structures were lifted from the substrate using a novel Neural Rosette Selection Reagent and subsequently replated onto poly-L-ornithine/laminin coated plates in neural progenitor proliferation medium to produce neural progenitor cells (NPCs). The cells were either maintained under these proliferative conditions for several passages or cultured in differentiation media to induce differentiation into neuronal subtypes and astrocytes. Our results indicated that 90-100% of the EBs cultured in Neural Induction Medium generated neural rosettes which contained Pax6, Sox1 and Nestin positive early-stage NPCs. The NPCs could be subcultured for multiple passages and differentiated into mature neurons and astrocytes as determined by the expression of TUJ1 and GFAP, respectively. This work describes a highly efficient protocol for the induction of neural progenitor cells and their progeny from hPSCs and may help to standardize the field.

Poster Board #49

Next Generation High-throughput Sequencing at Genomics Core Yale Stem Cell Center

Mei Zhong, Haifan Lin.
Yale University

Yale Stem Cell Center Genomics Core is one of the four core facilities of the Yale Stem Cell Center funded by the State of Connecticut and Yale University. The mission of the genomics core is to offer high level of expertise in next generation sequencing technology to supports stem cell research at Yale and cross the State of Connecticut. The genomics core is a hub of collaboration and a training site for stem cell researchers who wish to extend their research using genomic approaches. Next generation sequencing refers to high-throughput sequencing technologies parallelize the sequencing process, generating billions of bases of high-quality DNA sequence per run. Different platform constitute various strategies, but all rely on a combination of template preparation, sequencing and imaging, and genome alignment and assembly methods. A fundamental difference between the next generation sequencing and the traditional capillary sequencing is that sequencing reads are produced from fragment 'libraries' that have not been subject to the conventional vector-based cloning and amplification stages.
Yale Stem Cell Center Genomics Core offer standard services including DNA sequencing (DNA-Seq), Transcriptome analysis (RNA-Seq), Small RNA discovery (smRNA-Seq), Gene regulation & epigenetic analysis (ChIP-Seq) and cost effective Multiplex sequencing (4 samples in one lane). As part of technical development, the genomics core also offers collaborative research on projects to explore the sequencing technology to go beyond the illumina protocol limitation tailoring to our project's needs.

Experience with Diverse Cell Concentration, Retention, and Wash Applications for Use in Stem Cell Processing and Cell Therapy Processing

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[1]*Invetech,* [2]*KBI BioPharma*

Current cell separation and wash technologies have limited applications in cell therapy processing and manufacturing as they are optimized for either simple centrifugation, blood processing, or bone marrow aspirates. For example, most cell harvest processes are efficient in removing cells but not in maximizing viable cell density, viability, and recovery; parameters that are most important for stem cell processing or cell therapy manufacturing. Frequently, cells are retained by either centrifugation or filtration based devices. These approaches are often problematic. Conventional centrifugation based devices cause stress and nutrient deprivation to the cells in the pellet which results in low viability, while filtration based devices often suffer from issues such as clogging, retention of cell debris, and shear stress on cells.
Invetech in working with various partners over the past 10 years has been tasked with helping to develop commercial scale stem cell and general cell separation and wash apparatus. The problem has been that existing technology either stress the cells, killed cells, resulted in poor recovery from wash or could not be integrated into a functionally closed processing system. Invetech has successfully adapted several existing technologies and recently helped KBI BioPharma, Inc. design and develop a centrifugal fluid flow counter-force device for disposable cell recovery. The results of our efforts and examples of these apparatus will be exemplified, including the kSep™ by KBI Biopharma, Inc

High efficiency siRNA-mediated gene knockdown in human embryonic stem cells

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It is well known that human embryonic stem cells (hESCs) are difficult for genetic manipulation since their extremely low cloning efficiency. Here we developed a method that increased the efficiency by almost 40 folds than tranditional method. Applying this as a crucial step, we worked out an efficient double transfection system for siRNA targeting genes in hESCs, which can reproducibily knockdown endogenous pluripotent genes, Oct4 and Lin28, with more than 90% and highest 98% efficiency within 72 hours, and cells still kept normal proliferation capability, and no apparent enhancement of apoptosis. The knockdown system could be applied for different hESC lines and induced pluripotent cells (iPSCs) with more than 90% knockdown efficiency.

University of Connecticut-Wesleyan University Stem Cell Core in Year Five

Leann Crandall, Tiwanna Compton, Jung Park, Kristen Martins-Taylor, Jianle Wang, Xiaofang Wang, Stormy Chamberlain, Marc Lalande, Ren-He Xu.
UCONN-Wesleyan University Stem Cell Core

The UConn-Wesleyan Stem Cell Core resides in the UConn Stem Cell Institute, Cell and Genome Sciences Building at UConn Health Center. The Stem Cell Core lab was established in 2006, in response to the CT State Stem Cell Initiative. Thanks to institutional support by UCHC, and two core facility grants awarded by the state of Connecticut, the Stem Cell Core lab has been vital in supporting the rapidly growing, vibrant CT stem cell community. The Stem Cell Core serves laboratories at UCHC, UConn, Wesleyan, and throughout the entire state. To date: the Stem Cell Core has 1) trained over 140 researchers in human embryonic stem cell (hESC) and induced pluripotent stem cell (iPSC) culture methods; 2) hosted training workshops on neural, hematopoietic, and vascular differentiation, and on stem cell genomic analysis; and will hold an iPSC derivation workshop will be held this fall 3) derived 4 hESC lines, CT-1, -2, -3, and -4, which are registered at the NIH, and are available for distribution worldwide; 4) derived numerous normal or disease-associated human induced pluripotent stem cells (hiPSCs); and 5) distributed a variety of hESC and hiPSC lines to over 40 labs inside and out of Connecticut. Since 2007, the stem cell core has been providing these services through an institutionally supported Service Center. In 2010, the iPSC/Chromosome service center was formed to offer cytogenetic services, which will be administered at the Storrs campus of UConn by Dr. Judy Brown and Dr. Rachel O-Neill. Please visit the iPSCC booth for details of the services, which continue to include iPSC derivation service, quality control for iPSCs and hESCs, hESCs and iPSCs available for distribution, validated reagents, stem cell training courses and workshops. In the coming years, the Stem Cell Core will continue to provide our services to with the highest standards, and follow and develop new technologies, in keeping with our greater mission to support the advancement of stem cell research, including basic research as well as contribute to disease-fighting strategies

At the Yale Small Molecule Discovery Center

Janie Merkel, Laura Abriola, Peter Gareiss, Mariya Kolesnikova, Michael Norcia, Jay Schneekloth, Michael Kinch.
Yale University

Yale University has developed an interdisciplinary center to assist investigators in the pursuit of innovative therapeutic candidates. The Yale Small Molecule Discovery Center (YSMDC) specializes in the development of robust assays that are designed to be compatible with internal efforts to identify promising therapeutic candidates. In particular, studies at the YSMDC have identified novel chemical entities with the potential to elicit desired outcomes as assessed using objective model systems. The assays are initially developed by our academic partners. The Center then optimizes assay reliability and sensitivity to ensure that subsequent evaluation of compound libraries by the Center, or our commercial and academic partners, provides robust findings. The breadth of development includes both cell-based and purified protein assays. When combined with multiple libraries of small molecule compounds, it provides an efficient means to facilitate the isolation and prioritization of potential leads for future investigation. The data-driven approach of the YSMDC thereby identifies opportunities for assay improvement and follow-on testing of potential therapeutic candidates. Once developed, the small molecule screening approach utilizes a triage approach to identify early "hits" with subsequent efforts devoted to analyze specificity and potency. The determinants of a successful outcome can then be systematically evaluated by exploring related molecules that vary in the positions and chemical moieties around a common scaffold, which contributes towards the goals of optimizing efficacy, safety and drug-ability. By combining the industry experience of the Center staff with the innovative research of Yale investigators, the YSMDC provides a novel means to identify innovative new therapeutics.

Single Cell Analysis of Stimulated CellSensor® AP1-bla ME-180 Cells Using Biotrove Open Array System

Elena Grigorenko[1] Ronald V. Abruzzese[2], Laura Chapman[2], Kevin Munnelly[1] and Richard Fekete[2].
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When analyzing gene expression profiles from large numbers of cells the average profile may not be a true representation of the many different profiles that could exist in the cell population (ex., in different states of growth, differentiation or activation). The transcriptional variability of individual cells and any insight into the relationship between specific genes gets lost. One aspect of this emerging field that still needs to be developed is data analysis. Can you confidently identify sub-populations of single cells in a larger cell population? The data analysis of high sample numbers with a reduced number of targets is not as straightforward as when using many targets with a few samples (i.e. arrays). In this study we combined a well characterized CellSensor® AP1-bla ME-180 cell line, a modified Cells-to-Ct protocol and the Biotrove OpenArray® system to analyze up to 960 samples with 56 assays of interest. The CellSensor® AP-1-*bla* ME-180 cell line contains a beta-lactamase reporter gene under control of the Activator Protein-1 (AP-1) response element stably integrated into ME-180 cells. Epidermal Growth Factor (EGF) is a peptide that induces cellular proliferation through the EGF receptor. Proliferative effects of EGF signaling occur through several pathways, namely the activation of the ras and MAP kinase (MAPK) pathway. This in turn causes phosphorylation of transcription factors such as c-Fos to create AP-1 and ELK-1 that contribute to proliferation. Therefore, we investigated the effects of EGF stimulation on the gene expression profile of large and single cell populations by comparing untreated and EGF treated cells. The cells were sorted by FACSAria and an optimized workflow based on the commercially available kits, TaqMan® Gene Expression Cells-to-CT™ and TaqMan® PreAmp Cells-to- CT™ kits was used to investigate the expression levels of a limited number of genes in the MAPK pathway. We also tested the capability of the Biotrove OpenArray® to assay a larger number of genes in the MAPK pathway. The average Ct values were 10-fold different between the 100, 10 and 1 cell samples. Closer examination of the single cell samples shows heterogeneous populations; some genes being turned on or off in all samples, or just a change in the number of samples expressing a gene with no change in expression levels.

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Validation of a Combined Photoacoustic Micro-Ultrasound System for Estimating Blood Oxygenation

Andrew Heinmiller[1], Andrew Needles[1], Corina Bilan-Tracey[1], David Bates[1], Catherine Theodoropoulos[1], Desmond Hirson[1], Stuart Foster[2].
[1]*VisualSonics Inc,* [2]*University of Toronto*

Photoacoustic (PA) imaging can estimate the spatial distribution of oxygen saturation (sO_2) in blood, and be co-registered with B-Mode images of the surrounding anatomy. Here we focus on the development of a PA imaging mode on a commercially available array based micro-ultrasound (µUS) system that is capable of creating such images in real time. To validate the system, PA-derived in vivo sO_2 measurements were made and compared to expected sO2 values from the literature at various oxygen partial pressures (pO_2). The µUS system (Vevo LAZR, VisualSonics) was operated with a linear array transducer (LZ550, fc = 40 MHz) with integrated fiber optic bundles to either side. The rectangular bundles were bifurcated ends of a single bundle that was coupled to a tunable laser (Rainbow NIR, OPOTEK Inc., Carlsbad CA, 680-970 nm). The µUS system was synchronized with the laser and PA signals were acquired with a fluence < 20 mJ/cm^2 and beamformed in software. For sO_2 estimates, PA images were collected at 750 and 850 nm and parametric maps of sO_2 were displayed at 0.5 Hz. The concentration of oxygen mixed with the inhaled anesthetic was altered while sO_2 imaging was performed. A region of interest was created for the set of sO_2 images for each experiment to include a section of the jugular vein and the sO_2 was calculated based on this ROI for each selected pO_2 value. These sO_2 values were then compared to predicted sO_2 values based on the measured pO_2. Changes in pO_2 values and sO_2 values correlated well with alterations in inhaled O_2 concentration and PA-derived sO_2 values correlated well with predicted values. Photoacoustic imaging with the Vevo LAZR system can provide a good estimate of intravascular sO_2.

Stable Expression of Transgenes at a Defined Locus in Human Embryonic Stem Cells

Pete Conway[1], Chad C. Macarthur[1], Haipeng Xue[1], Dennis Van Hoof[2], Pauline Lieu[1], Andrew Fontes[1], Bhaskar Thyagarajan[1], Jon D. Chesnut[1], Mahendra S. Rao[1], Pauline Lieu[3].
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Human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) are invaluable tools to study gene function in humans. Hence, the identification of a suitable genomic locus in hESCs and iPSCs, and the creation of platform pluripotent lines which ensure efficient, reliable, and stable genetic engineering are in great need. Here, we used PhiC31 integrase mediated recombination technology to target such a locus at chromosome 13 in the genome of a commonly used hESC line, and inserted specific attachment sites to allow for precise single-copy retargeting at the previously mapped locus. In order to minimize possible genomic positional effects, and to mitigate potential silencing issues in gene expression, our integrating constructs were flanked by cHS4 double insulator sequences. To characterize this locus, we retargeted one copy of the GFP cassette, driven by one of two broadly expressed promoters; CAG or EF1α. We found that in the engineered hESC lines, for both CAG-GFP and EF1α-GFP promoter- reporter pairs, constitutive expression at the chromosome 13q32 locus was maintained during long term culture and in directed differentiation processes towards various cell types, including diverse types of neurons, muscle cells, pancreatic progenitor cells and hepatocytes. The creation of multiple hESC platform lines at the chromosome 13q32 locus provides a valuable tool for lineage development study, gain- and loss-of-function experiments, and human disease modeling. The platform lines are useful for both basic biology and translational research for regenerative medicine.

Detection and Quantification of mRNA Transcripts in Single Embryonic Stem Cells by OpenArray® and Digital PCR

Fred Kolling IV[1], Elena Grigorenko[2], Sunali Patel[2], Kevin Munnelly[2], Craig Nelson[1].
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There is increasing evidence that pluripotent embryonic stem cell cultures (ESCs), once thought to be a uniform cell type, are in fact highly heterogeneous, especially in terms of RNA expression. Until recently, understanding the dynamics of both miRNA and mRNA expression within a cell population has been severely limited by the lack of an efficient and effective means to assay gene expression within individual cells. Here we demonstrate our success using the Taqman™ chemistry on Open Array platform (Life Technologies, Inc) to easily and robustly analyze miRNA and mRNA expression in single embryonic stem cells. Open Array results are consistent with our established single cell analysis platform, confirming the robust reproducibility of the Open Array system, and establishing the technology required for high throughput single cell profiling experiments. In our most recent studies we confirm highly heterogeneous expression of key developmental control genes in ESC and demonstrate heterogeneous expression of several miRNAs, adding a new dimension to our understanding of cell type complexity in ESC cultures. In addition, we utilized cutting-edge digital PCR (dPCR) technology to quantify the number of transcripts present in each cell, providing an unprecedented level of resolution to single cell transcript analysis. Single mouse embryonic stem cells (mESCs) were collected via FACS sorting into 96 well plates containing Single Cell-to-Ct® Lysis Buffer with subsequent DNA treatment, RT and preamplification performed using either gene-specific or miRNA Megaplex® primers and samples were sent to Life Technologies for analysis. In total we analyzed the expression of 64 mRNA transcripts in 48 single cells and 61 miRNAs in 27 cells using the Open Array® platform. For dPCR, cells were lysed following FACS and sent to Life Technologies where RNA was reversed transcribed and the entire RT reaction was applied to the Open Array®. dPCR was used to quantify Nanog and Gapdh transcripts for pools containing between 1-20 cells and per-cell transcript levels were consistent across all samples. Our experience with the Open Array® platform and its dPCR capability has dramatically increased our throughput, providing a flexible and cost-effective means of analyzing gene expression in single cells without compromising the reliability and sensitivity of our assay.

Scalable Neural Stem Cells Produce Robust Neural Model

Marsha Roach[1], Richard Malavarca[1], Angela Cacace[2] Myles Fennell[2].
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In neuroscience drug discovery today there is a major unmet need for different types of mature human neurons that can be utilized to investigate drugs for the treatment of neurological disorders such as Alzheimer's Disease. Consequently, pharmaceutical companies are in search of more physiologically relevant pre-clinical models that can reliably predict the safety and efficacy of new pharmacological agents. With increasing attrition rates and associated development costs of new compounds in clinical trials, a great need exists to improve the early-stage selection process of candidate compounds in order to limit the number of costly failures in late phase clinical trials. Thus we have developed stem cell-derived neural models to meet that need.

Recent advances in our stem cell technology and media formulations have led to the development of a scalable human neural stem/progenitor cell (NSPCs) culture system that enables neuroscience researchers to produce mature human neurons in a 384-well format that is compatible with automation and high throughput screening. Here we will demonstrate that human NSPCs can be expanded to a scale greater than 109 cells that can be stored in a cryo-bank so that a screen of full file chemical libraries can be performed from one bank of cells. Upon thawing, the human NSPCs can be reproducibly differentiated into mature neural populations that are at least 80-90% neurons with mature synapses.

Rare Event detection using Acoustic Cytometry

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Detection of rare events includes populations of cells comprising less than 5% of the total cells, which includes the detection of stem cells, minimal residual disease, tetramers, NKT cells and fetomaternal hemorrhage. Analysis of rare cell populations requires the collection of high numbers of events in order to attain a reliable measure of accuracy, leading to long acquisition times. Acoustic cytometry is a new technology that allows dilute samples to be processed quickly. Conventional cytometers attain a static volumetric ratio of sheath to sample flow of typically greater than 100. By combining acoustic and hydrodynamic focusing, ratios can be reduced by several orders of magnitude and sample injection rates can be increased up to 6 fold (1ml/min) compared to pressure driven cytometers, allowing higher throughput with dilute samples. The collection times and percentage of rare events detected of the Applied Biosystems® Attune™ Acoustic Focusing Cytometer are compared to a conventional cytometer that uses hydrodynamic focusing.

Global analysis of genes involved in neural commitment and differentiation of human embryonic stem cells using pooled shRNA libraries coupled with next-generation sequencing

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sh or siRNA knockdown of specific genes is a powerful screening tool for gene function. Here by using pooled shRNA library and cutting edge deep sequencing technology, we have dramatically expand the ability of this approach from single gene to genome-wide gene function studies. We have quantitatively determined the enrichment and drop out of shRNAs in cells of various experimental paradigms. The deep-sequencing method has several advantages over the array hybridization, including a very wide dynamic range for detecting shRNAs of differing abundance, and accurate detection including the discovery of mutant shRNAs that might confound analyses.

We used the model of undifferentiated human ES cells to transduce with pools of human shRNA lentiviral library targeting the entire genome at MOI of 0.3 for each condition. Each pool contained ~ 8000 distinct shRNAs where 4-5 shRNAs target the same gene. The uninfected cells were removed by puromycin and the rest of the cell population was divided into two experimental arms: either to propagate in conditions that select for pluripotency and analyze the time course of this process or to differentiate the cells into neuronal precursors/progenitors. At the end of differentiation the cells were sorted for the neural surface marker NCAM and binned.

DNA were isolated from NCAM+ and - populations, the shRNA inserts were recovered by PCR and analyzed by Solexa/Illumina sequencing. Most shRNAs were recovered in the same relative levels in both DNA preparations but a significant number were relatively enriched in the cells that had grown for a shorter time. This experiment shows the technical feasibility of the experiments but also indicates the difficulties when there is only a single shRNA per gene, as it would be tedious to have to work up each putative shRNA separately.