# Table of Contents

Welcome ................................................................................................................................. 2
Acknowledgments .................................................................................................................. 3
Organizing Committee Members & Support Staff ............................................................... 4
Campus Map and Building Layouts ...................................................................................... 5
Agenda .................................................................................................................................. 11
Plenary, Breakout Session, and Technology Fair Abstracts .................................................. 14
Poncz Hub Site 01 .................................................................................................................. 15
Thomson Hub Site 02 ............................................................................................................. 21
Friedman Hub Site 03 .......................................................................................................... 27
Wu Hub Site 04 .................................................................................................................... 32
Torok-Storb Hub Site 05 ...................................................................................................... 44
Krasnow Hub Site 06 .......................................................................................................... 48
Weissman Hub Site 07 ......................................................................................................... 52
Daley Hub Site 08 ............................................................................................................... 57
Bernstein Hub Site 09 ......................................................................................................... 62
Cooke Hub Site 10 ............................................................................................................. 65
Hatzopoulos Hub Site 11 .................................................................................................... 70
Schneider Hub Site 12 ........................................................................................................ 74
Scadden Hub Site 13 ......................................................................................................... 79
Morrisey Hub Site 14 ......................................................................................................... 83
Srivastava Hub Site 15 ....................................................................................................... 88
Garry Hub Site 16 .............................................................................................................. 91
Parker Hub Site 17 ............................................................................................................ 99
U01 Hub Site Principal Investigators .................................................................................. 107
Meeting Participants .......................................................................................................... 109
Public Transportation from Airports to Stanford University ................................................ 125
Nearby Restaurants ......................................................................................................... 126
Taxi Service ....................................................................................................................... 129
Notes .................................................................................................................................. 130
Institutional Supporters .................................................................................................... 139
Index .................................................................................................................................. 140
September 13, 2014

Dear Colleagues and Friends,

On behalf of the Stanford University Research Hubs of the NHLBI Progenitor Cell Biology Consortium (PCBC), we would like to welcome you to Stanford University and School of Medicine for the 6th Annual PCBC Meeting. We hope to provide you with a welcoming and comfortable environment that will promote collegiality and collaborative interactions, as PCBC investigators review the past year’s progress and set plans for the future.

In planning this meeting, our goal is to stimulate robust scientific discussion and exchange among PCBC investigators and post-doctoral fellows and trainees so that new collaborations between hubs may be initiated from these encounters. We have developed a three full day agenda starting with the first day devoted to Fellows Only Session for presentations by post-doctoral fellows and graduate students. This is followed by two days of Research Hub reports, four breakout sessions, and reports from various different PCBC task forces and committees, and an optional morning following the meeting of a hands-on course on single cell RNA-Seq technology.

In the spirit of advancing stem cell science for heart, lung, and blood diseases, we have focused this annual meeting on novel technologies as the overarching theme. Towards this end, we initiated a new Technology Fair session where each hub is invited to showcase a new cutting-edge technology and we’ve incorporated a special didactic session on Single Cell RNA-Seq Analysis to be given by Dr. Stephen Quake from the Stanford Hub plus a new hands-on course on single cell RNA-Seq taught by members of the Quake and Krasnow labs.

Beyond the stimulating scientific presentations and discussions, we invite our colleagues to explore the iconic Stanford campus and the vibrant community in Downtown Palo Alto. A number of modern and classical art museums are open to visitors free of charge and a guided tour of the Stanford campus is available. Following the poster session on Monday, we have scheduled shuttle buses that will transport you to Downtown Palo Alto where a wide variety of restaurants can be found and bars with lively crowds. You might even recognize a few notable locals from companies like Google and Facebook.

We would like to thank the Dean’s Office of the Stanford University School of Medicine for supporting this meeting and everyone who has contributed time and effort to make this a successful event.

Thank you all for coming to Stanford!

Sincerely,

Irving L. Weissman, MD
Professor and Director
Institute for Stem Cell Biology and Regenerative Medicine

Mark Krasnow, MD, PhD
Professor and Chair
Department of Biochemistry
Director, Wall Center for Pulmonary Vascular Disease

Joseph C. Wu, MD, PhD
Professor of Medicine (Cardiology) and Radiology
Director, Stanford Cardiovascular Institute

Irving L. Weissman, MD
Professor and Director
Institute for Stem Cell Biology and Regenerative Medicine

Mark Krasnow, MD, PhD
Professor and Chair
Department of Biochemistry
Director, Wall Center for Pulmonary Vascular Disease

Joseph C. Wu, MD, PhD
Professor of Medicine (Cardiology) and Radiology
Director, Stanford Cardiovascular Institute
Acknowledgments

The National Heart, Lung, and Blood Institute (NHLBI) Progenitor Cell Biology Consortium Administrative Coordinating Center (NHLBI PCACC), Stanford University investigators, and affiliated consortium investigators, organized this 6th Annual PCBC Meeting at Palo Alto, CA.

The Consortium extends gratitude to Drs. Irving Weissman at the Institute for Stem Cell Biology and Regenerative Medicine, Mark Krasnow at the Department of Biochemistry and the Wall Center for Pulmonary Vascular Disease, and Joseph Wu at the Stanford Cardiovascular Institute for their generous support and hospitality in hosting the annual meeting.

Special acknowledgment also goes to Mr. David Preston (Stanford Cardiovascular Institute), Ms. Cynthia MacQuarrie (Institute for Stem Cell Biology and Regenerative Medicine), Ms. Andrea Lefever (University of Maryland, Baltimore), Ms. Elizabeth Casher (University of Maryland, Baltimore), and Ms. Ling Tang (University of Maryland, Baltimore) for work on meeting logistics.

The Consortium further extends appreciation to Drs. Jared Churko (Stanford University), Paige DeBenedittis (Vanderbilt University), Shu Meng (Houston Methodist Hospital Research Institute), and Lehanna Sanders (Vanderbilt University) for their energetic efforts in organizing the Fellows Only Session (FOS) with guidance from Drs. Beverly Torok-Storb (Chair of the PCBC Skills Development Committee) and Randy Daughters (Deputy Chair of the PCBC Skills Development Committee).

Please direct any questions or concerns that may arise in planning your participation in the 6th Annual PCBC Meeting to the staff of the PCBC Administrative Coordinating Center:

Elizabeth Casher;
Andrea Lefever;
Michael McCreery;
Lynn Schriml;
Ling Tang; and
Michael Terrin.
## Organizing Committee Members & Support Staff

<table>
<thead>
<tr>
<th>Name</th>
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<tr>
<td>Denis Buxton</td>
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<td>Liz Casher</td>
<td>University of Maryland School of Medicine</td>
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<td>Jared Churko</td>
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<td>Joseph Wu</td>
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<td><strong>Sean Wu</strong></td>
<td>Stanford University</td>
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The 6th Annual PCBC Meeting will take place in the Li Ka Shing Center (LKSC) and Lorrey I. Lokey Stem Cell Research building. Plenary sessions and the reception/poster session will take place in Paul Berg Hall (Rooms A & B plenary session, Room C reception/poster session). Breakout sessions will take place in smaller rooms in the LKSC and Lorrey I. Lokey Stem Cell Research building which is adjacent to the LKSC. The PCBC Steering Committee meeting will take place in a smaller room in the LKSC.

### Campus Map and Building Layouts

The Stanford Campus Map includes:
- Li Ka Shing Conference Hall – location for the main meeting (plenary and breakout sessions)
- Campus parking options identified
- Shuttle pick-up and drop-off identified
- Points of interest identified

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<td><strong>Stanford Campus Map:</strong></td>
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<td><strong>Li Ka Shing Center (LKSC) – ground floor plan</strong></td>
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<td>- Breakout session rooms</td>
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<td><strong>Lorrey I. Lokey Stem Cell Research Building</strong></td>
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Li Ka Shing Center (LKSC)

Floor Plan

Conference Center
at the Li Ka Shing Center
Stanford School of Medicine

www.progenitorcells.org
Directions to the Lorry Lokey for Breakout Sessions:

When exiting Paul Berg Hall, take a right and go outside and down the outside stairway exiting the LKSC Building.

Turn right.

The Lorry Lokey Building, labeled as such, is the first building on your left…about a 2 minute walk.
Agenda

6th Annual PCBC Meeting
September 29-30, 2014
Li Ka Shing Center, Stanford University, Stanford, CA

Monday, September 29, 2014 (Paul Berg Hall A & B, Second Floor LKSC)

08:30 AM  Registration
09:00 AM  PCBC Welcome and Opening Remarks
   Victor Dzau
09:05 AM  Report from the NHLBI
   Denis Buxton
   •  PCBC Initiatives and Plans for 2015
   •  (Cores, Ancillary/Collaborative/Pilot Studies)
   •  Progress Reports
   •  NHLBI Initiatives Related to PCBC
   •  Future Plans for the PCBC
09:20 AM  Report from the Administrative Coordinating Center
   Michael Terrin
   •  Administration
   •  Web Site and Document Management System
09:30 AM  Research Hub Report (Torok-Storb/Poncz Research Hub)
   “Inducible GATA1 Suppression as a Novel Strategy to Expand Physiologic Megakaryocyte Production”
   •  Ji-Yoon Noh
10:00 AM  Research Hub Report (Srivastava/Wu Research Hub)
   “Transcriptomic Differences between Human Induced Pluripotent Stem Cell Derived Cardiomyocytes and the Adult Human Myocardium”
   •  Jared Churko
10:30 AM  Research Hub Report (Krasnow/Weissman Research Hub)
   “Interchangeable fates of osteogenic and chondrogenic progenitors revealed by comprehensive lineage-mapping of multipotent skeletal stem cells”
   •  Charles K.F. Chan
11:00 AM  Break
11:30 AM  Breakout Session I*
12:00 Noon Breakout Session II*
Agenda (continued)

12:30 PM  Lunch/Steering Committee Meeting (Room LK 005, First Floor LKSC)

01:00 PM – 1:50 PM  Single cell RNA-seq Didactic Session (Paul Berg Hall A & B)
  • Steve Quake

Monday, September 29, 2014 (Paul Berg Hall A & B, Second Floor LKSC)

02:00 PM  Research Hub Report (Cooke/Friedman Research Hub)
  “Optimized Rewiring of WNT and ERK Pathways is Sufficient for Stable Conversion of Human Pluripotent Stem Cells to a Naïve Ground State of Pluripotency”
  • Ludovic Zimmerlin

  “POU3F2 Regulates Endothelial Cell Differentiation and Vascular Development”
  • Gianfranco Matrone

02:30 PM  Break (and group photograph in front of LKSC)

03:00 PM  Research Hub Report (Hatzopoulos Research Hub)
  “GREM2 is a BMP Antagonist that Directs Human iPS Cell Cardiac Differentiation”
  • Jeffery Bylund

03:30 PM  Research Hub Report (Garry/Thomson Research Hub)
  “Lineage Reprogramming Of Mouse Fibroblasts to Proliferative and Multipotent Induced Cardiac Progenitor Cells by Defined Factors”
  • Pratik A. Lalit

4:00 PM  Research Hub Report (Morrisey/Bernstein Research Hub)
  “The Epigenetic and Transcriptional Landscape of Mesoderm Progenitor Cells Identifies Novel Regulators That Direct Cardiac versus Hemogenic Endothelial Fate”
  • Nathan J. Palpant

04:45 PM  Breakout Session III*

05:15 PM  Breakout Session IV*

05:45 PM  Poster Session/Reception (Paul Berg Hall C, Second Floor LKSC)

Tuesday, September 30, 2014 (Paul Berg Hall A & B, Second Floor LKSC)

08:30 AM  Registration Continued

08:45 AM  Panel and Open Forum on Novel Concepts and Controversies in Stem Cell Biology
Agenda (continued)

09:15 AM  CCTRN
  • Robert Simari

09:45 AM  Research Hub Report (Scadden/Schneider Research Hub)
  “Prevention of Muscular Dystrophy in Mice by CRISPR (Clustered
  Regularly Interspaced Short Palindromic Repeat)/Cas9 (CRISPR
  Associated Protein 9)-mediated Gene Editing”
  • Chengzu Long

10:15 AM  Technology Fair (Paul Berg Hall C, Second Floor LKSC)

12:00 Noon  Lunch

01:15 PM  Research Hub Report (Parker/Daley Research Hub)
  “Flow-induced Protein Kinase A / CREB Pathway Acts via BMP
  Signaling to Promote AGM Hematopoiesis”
  • Peter Geon Kim

01:45 PM  Report on the Fellows Only Session of September 28, 2014

02:15 PM  Break

02:30 PM  Skills Development Committee
  • Beverly Torok-Storb

02:50 PM  Core Reports
  • Bioinformatics Core – Larsson Omberg
  • Cell Characterization Core – Punam Malik
  • RNA Core – John Cooke

03:20 PM  Bioinformatics Committee
  • Antonis Hatzopoulos

03:50 PM  Working Group Reports
  • Hematopoiesis – Irv Bernstein
  • Cardiovascular – Joseph Wu and Sean Wu
  • Pulmonary – Ed Morrisey
  • Technology – Mark Krasnow and John Cooke

04:30 PM  Concluding Business and Adjourn
  • Victor Dzau
Plenary, Breakout Session, and Technology Fair Abstracts

Plenary Session, Breakout Session and Technology Fair Abstracts are organized by Hub Site. Within Hub Site abstracts are organized alphabetically by last name of the first author. Specifications of each abstract as being presented in the Plenary Session, Breakout Session, or Technology Fair are to be found in the “Index”, pages 140-146.
Poncz Hub Site 01
ABSTRACT # 1

A Doxycycline-Inducible System for Gene Correction of Patient-Derived Induced Pluripotent Stem Cells

Fabian L. Cardenas, Xiuli Sim, Deborah L. French, Paul Gadue

University of Pennsylvania, Philadelphia, PA

Patient-derived induced pluripotent stem cells (iPSCs) are valuable tools for studying human development and modeling disease. To fully dissect a phenotype, correction and expression of genes involved in disease pathogenesis are required. A common method used for genetic correction is virus-driven transgene expression. This method can be deleterious resulting in random insertions leading to off-target effects such as silencing of the transgene during cell differentiation and high copy number resulting in non-physiological expression of the transgene. In addition, iPSC lines with subtle phenotypes can be difficult to study due to clonal variation and experimental variability inherent in directed differentiation protocols. Here, we describe a simple and efficient method to generate a doxycycline (dox)-inducible system for the expression of transgenes in human iPSCs. The power of this system is to control the timing and dosage of gene expression using dox, thus eliminating the influence of clonal variation and genetic background in the analysis of a phenotype.

To validate this technology, we generated a homozygous knock-in human embryonic stem cell (hESC) line with dox-inducible expression of GFP. This was achieved in one step using lipid transfection of a zinc finger nuclease targeting both alleles of the “safe harbor” locus, AAVS1. One allele was targeted with a construct driving the constitutive expression of the reverse tet-transactivator (rtTA). The second allele was targeted with the doxycycline-inducible tet-response element (TRE) driving the expression of GFP, or any gene of interest. Hematopoietic differentiation of this hESC line to progenitors and megakaryocytes showed dox-inducible GFP expression in both cell populations. The advantages of this system are that 1) it is extremely efficient with the percentage of double-targeted clones ranging between 20-60%, 2) the level of transgene expression is proportional to dox concentration allowing titration of gene expression, and 3) it allows experimentation using a single cell line, removing clonal and experimental variation in directed differentiation protocols. This technology can be broadly applied to gene correction of patient-derived iPSCs for studies of blood or other disorders and gene function.
ABSTRACT # 2

Comparative Analysis of Infused “Static”, ex vivo-generated Platelets vs. Infused Megakaryocytes-generated Platelets: A Cautionary Tale

Danuta Jarocha, Yuhuan Wang, Vincent Hayes, and Mortimer Poncz

University of Pennsylvania, Philadelphia, PA

Ex vivo-generated (EV) platelets beginning with ES or iPSCs or HPCs may have clinical utility over donor-derived (DD) platelets, and efforts to produce such EV-platelets have been pursued under static megakaryocyte (Meg) culture conditions. Success in generating these has been reported, even demonstrating EV-platelet incorporation into growing thrombi in mice. We have pursued an alternative strategy for thrombopoiesis using EV-Megs, grown from either human adult HPCs or from iPSCs or fetal livers, and directly infusing them into NSG mice. These studies were based on our prior observation that infused murine EV-Megs into wildtype mice are entrapped in the pulmonary bed and over 1-4 hours release a wave of functional platelets. We now show that infusion of human EV-Megs do the same in NSG mice, but resulting in two different pools of derived platelets: (1) A pool of young (as determined by thiazole orange staining) platelets having the same bell-shaped size distribution as seen after infusion of human DD-platelets. These platelets take several hours to appear, but have the same half-life as DD-platelets. These platelets are derived from the infused EV-Megs and were termed in vivo-generated (IV)-platelets. (2) A second pool of mostly older platelets was present that originated during the static growth of the EV-Megs, and these EV-platelets varied widely in size and age. Initially, these platelets accounted for a third of all the human platelets seen. Unlike IV-platelets, EV-platelets are immediately present and circulate with a markedly short half-life of 2-3 hours unless the recipient NSG mice were pre-treated with clodronate-ladened liposomes to delete macrophages. Rapid removal of EV-platelets by macrophages is due to their being preactivated as determined by surface p-selectin expression. The IV-platelets also had very limited further responsiveness to convulxin activation. On the other hand, human IV-platelets were quiescent prior to agonist stimulation in whole mice blood and responded strongly to agonist, similar to human donor-derived platelets infused into NSG mice. The IV-platelets were also selectively incorporated into cremaster arteriole laser injury thrombi over EV-platelets. These findings set a standard on how to judge the potential clinical value of platelets derived from EV-Megs.
Inducible GATA1 Suppression as a Novel Strategy to Expand Physiologic Megakaryocyte Production


Children’s Hospital of Philadelphia, Philadelphia, PA

ES and iPS cells represent potential sources of megakaryocytes and platelets for transfusion therapy. However, it is prohibited by low yields of platelet-releasing megakaryocytes using current ES/iPS cell differentiation protocols. Mutations in the mouse and human genes encoding transcription factor GATA1 cause accumulation of proliferating, immature megakaryocytes. Previously, we reported a self-renewing hematopoietic progenitor G1ME that were generated from Gata1-null murine ES cells. However, terminal maturation of GATA1-rescued megakaryocytes was aberrant, presumably due to non-physiologic restoration of GATA1 by retrovirus. We assessed the hypothesis that physiologic restoration of GATA1 in Gata1-null ES cells can improve terminal megakaryopoiesis. We engineered wildtype murine ES cells to express doxycycline (dox)-regulated Gata1 short hairpin (sh) RNAs and developed a strategy for Gata1-blockade that upon release, restores physiologic GATA1 levels in megakaryocytes. In vitro hematopoietic differentiation of Gata1 shRNA-expressing ES cells with dox and thrombopoietin (TPO) produced immature hematopoietic progenitors, termed G1ME2 cells, which replicated continuously for more than 40 days, resulting in ~1013-fold expansion, while control scramble shRNA-expressing ES cells underwent senescence in 7 days. Upon dox withdrawal with TPO alone, endogenous GATA1 expression was restored, and the G1ME2 cells formed mature megakaryocytes in 5-6 days, as determined by morphology, ultrastructure, gene expression, CD42b surface expression, DNA ploidy and proplatelet formation. In clonal methylcellulose assays, dox-deprived G1ME2 cells differentiated into erythroid and megakaryocytic colonies with multi-lineage cytokines (EPO, TPO, GMCSF and IL3). Importantly, G1ME2-derived megakaryocytes generated functional platelets in vivo that were actively incorporated into growing arteriolar thrombi at sites of laser injury and subsequently expressed the platelet activation marker p-selectin. In conclusion, precise timing and level of a transcription factor is required for proper terminal megakaryopoiesis and the current strategy provides a proof-of-principal for production of functional platelets in clinically relevant numbers.
ABSTRACT # 4

A Transcriptome Profiling Approach to Define the Stages of Megakaryocyte Maturation

Xiuli Sim, Prasuna Paluru, Lin Lu, John Tobias, Deborah L. French, Paul Gadue

University of Pennsylvania, Philadelphia, PA

Megakaryocytes (MKs) are specialized hematopoietic cells that produce platelets, which are important for hemostasis. Defined surface markers are used to identify MKs, such as the fibrinogen receptor, αIIbβ3 and the von Willebrand factor receptor (CD42). During MK maturation, the αIIbβ3 receptor is expressed first followed by expression of CD42. The changes in gene expression during MK maturation after the expression of CD42 are not well understood. MKs are difficult to study as this population is extremely rare in the bone marrow at only 1 in 10,000 cells. It is our goal to define the transcriptional signature of MKs at different stages of maturation, in order to better understand megakaryopoiesis.

Here we describe two populations of CD42+ late stage MKs that arise during the differentiation of primary human CD34+ hematopoietic progenitors. These two populations have distinct size and granularity, hence we term them the CD42+ high granular MKs and the CD42+ low granular MKs. Through cell sorting and time course experiments, we demonstrate that the low granular MKs arise first and mature into the high granular MKs. As the MKs mature, they lose surface expression of CD43, a pan-hematopoietic marker. Of interest is the finding that these two MK populations have distinct expression patterns of pro-angiogenic and anti-angiogenic proteins that are found in the alpha granules of platelets. Specifically, we detect an increase in anti-angiogenic protein and a decrease in pro-angiogenic protein expression intracellularly as the low granular MKs mature into the high granular MKs. This may be due to the secretion of pro-angiogenic proteins by the high granular MKs, a possible phenomenon to attract and promote blood vessels sprouting prior to platelet release.

To better characterize MK maturation, we performed a gene expression microarray to compare the transcriptome of the mature high granular MKs and the less mature low granular MKs. Interestingly, many of the most highly upregulated transcripts in the mature population are long non-coding RNAs and small nuclear RNAs, with unknown functions in megakaryopoiesis/thrombopoiesis. Most genes that are known to be associated with megakaryocytes/platelets show little difference in expression between the low and high granular group. Currently, we are undertaking more detailed analyses to understand the gene expression differences between the low and high granular populations and whether there is functional significance associated with these differences. Detailed staging of MK maturation may allow us to accurately isolate the specific stage where platelet production is the most robust. We may be able to find methods to confine MKs to this stage to enhance the efficiency of in vitro platelet production for therapeutic purposes.
ABSTRACT # 5

Using an iPSC Model of Jacobsen Syndrome to Study the Importance of FLI1 and ETS1 in Megakaryopoiesis

Karen Vo, Deborah French, Spencer Sullivan, Mortimer Poncz

University of Pennsylvania, Philadelphia, PA

Jacobsen syndrome is an inherited autosomal dominant deletion in chromosome 11q and is associated with dysmegakaryopoiesis and a macrothrombocytopenia termed Paris-Trousseau syndrome (PTSx). The hemizygous deletion includes the closely-linked Friend leukemia integration 1 (FLI1) and v-ets avian erythroblastosis virus E26 oncogene homolog 1 (ETS1) transcription factors that are important during megakaryopoiesis. The goal of our study is to better understand megakaryopoiesis by studying the roles of FLI1 and ETS1 in both normal and perturbed (patient and knock out/knock in) systems. We hypothesize that the observed macrothrombocytopenia seen in PTSx is due to a combination of FLI1 and ETS1 haploinsufficiency. We have established iPSC lines from one PTSx patient and derived from this a FLI1 overexpressing (OE) line driven by the platelet-specific promoter for the Gp1ba murine gene. In parallel, we established a healthy control line, a control-derived FLI1-OE line, and a TALEN gene editing FLI1+/- line. Megakaryocytes derived from these iPSCs (iMega) have been characterized. As expected, FACS analysis of megakaryocyte markers of patient iMega shows very low CD41 and CD42 expression. Increased CD41 and CD42 were observed in the patient-derived FLI1 OE iMega, however, not to the level of control iMega. This suggests there may be a need for ETS1 overexpression as well as FLI1 in the PTSx iMega to compensate for its hemizygous loss. Of note, control-derived FLI1+/- iMega have similar levels of CD41 and CD42 compared to PTSx FLI1 OE, further supporting that both FLI1 and ETS1 need to be present to observe the degree of loss of megakaryopoiesis seen in PTSx. Additionally, iMega from the control, control-derived FLI1 OE and FLI1+/- lines were classified as either mature or intermediate according to intensity of CD41 and CD42 expression via FACS analysis, and following cell sorting and subsequent qRT-PCR, we found that FLI1 is more highly expressed in the mature population compared to the intermediate population in all three lines. In the intermediate megakaryocyte population of the FLI1+/- cells, there was little to no FLI1. The FLI1+/- intermediate population could fit a haploinsufficiency model in which the majority of cells are stuck in development, while FLI1+/- mature megakaryocytes represent those few cells that had sufficient FLI1 expression to get through a critical developmental point. In conclusion, our findings suggest a role for both FLI1 and ETS1 in the disturbance of megakaryocyte maturation in PTSx. At least for FLI1, cells can reach an immature megakaryocyte state with low to no levels of expression, but need a large increase in expression to complete megakaryocyte differentiation. Additional iPSC lines manipulating the level of ETS1 alone and concurrently with FLI1 are needed to complete these studies.
Thomson Hub Site 02
ABSTRACT # 6

Artificial Transcription Factors to Control Cell Fate Choices

Asuka Eguchi, Graham S. Erwin, José A. Rodríguez-Martínez, and Aseem Z. Ansari
University of Wisconsin, Madison, WI

Artificial transcription factors (ATFs) can be used to identify master regulators of cell fate choices. ATFs are precision-targeted proteins or small molecules that bind DNA specifically and regulate transcription. Modular design principles allow us to tailor the function, binding specificity, and appropriate binding partners of the ATF to suit one’s experimental needs.

Regulation of repressed genes
Small molecule-based genome readers provide an attractive means to regulate transcription without the introduction of genetic material into cells. Polyamides, made of N-methylpyrrole and N-methylimidazole rings, can be rationally designed to bind DNA in a sequence-specific manner. Unlike proteins, polyamides can target chromatinized and methylated sites in the genome. We developed a technique called crosslinking of small molecules for isolation of chromatin (COSMIC) to identify the genomic targets of polyamides.

Transcriptional network discovery
Genetically encoded ATFs can be used to build a library consisting of millions of different members, each of which targets a different DNA sequence. Such a library can be used to discover key nodes in transcriptional networks, which trigger cell fate changes. We made a zinc finger ATF library with the complexity to bind an array of 9-bp sequences in the genome and activate transcription. By using this forward genetics approach, we identified ATFs that can direct the pluripotency network and the hematopoietic network.

Target site identification
To identify functional binding sites in the genome, we developed Cognate Site Identifier (CSI). This assay permits us to measure the binding preferences of transcription factors in a comprehensive sequence space and reveals ranges of low to high affinity binding sites in an unbiased manner. The spectrum of binding specificities can be displayed as Sequence Specificity Landscapes (SSLs), which can be used to identify differences between closely related TFs. These differences are exploited in the design of precision-tailored ATFs. In conclusion, we describe a suite of tools to accurately annotate regulatory elements in the genome and deliver ATFs exogenously to trigger transcriptional networks with exquisite control.
ABSTRACT # 7

Precision-Targeted Small Molecule Genome Readers and Epigenetic Remodelers to Regulate Transcriptional Networks

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Targeting the genome with sequence-specific synthetic molecules is a major goal at the interface of chemistry, biology, and regenerative medicine. Many DNA-binding molecules exist, such as zinc fingers (ZFs), transcription activator-like effectors (TALEs) and RNA-guided proteins (CRISPR-Cas). However, these scaffolds have limitations in areas such as programmability and the ability to access methylated and chromatinized DNA. Engineered small molecules, called polyamides, can be rationally designed to target specific DNA sequences with exquisite precision, and they retain their affinity when binding to methylated and chromatinized DNA without leaving a genetic footprint. Moreover, they can be used to regulate gene networks. We have synthesized a focused library of polyamides that can target many different DNA sequences. To understand how polyamides engage the genome, we developed a method to identify polyamide binding sites in human cells, which revealed a new design principle to deploy synthetic molecules to effectively target desired genomic sites. Importantly, polyamides can be attached to chromatin remodelers to make the chromatin environment more accessible to transcription factors at desired genomic loci. A polyamide attached to the HDAC inhibitor, SAHA, potently activates Oct4 and Nanog in mouse embryonic fibroblasts. We will present recent advances of polyamides as powerful tools to regulate transcriptional networks and reset the cell’s homeostatic state to permit the activation of cell fate-defining genes in a programmed manner.
ABSTRACT # 8

Lineage Reprogramming Of Mouse Fibroblasts to Proliferative and Multipotent Induced Cardiac Progenitor Cells by Defined Factors


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Reprogramming somatic cells to cardiac progenitor cells (CPCs), which may be more favorable for cell therapy because of their proliferative properties and multipotency, has not been accomplished. We hypothesized that utilizing the knowledge of cardiovascular development and TF-mediated reprogramming, mouse fibroblasts can be directly reprogrammed to induced cardiac progenitor cells (iCPCs). We cloned a library of 22 cardiac-related genes into a doxycycline inducible lentivirus vector. Fibroblasts used for reprogramming were derived from an Nkx 2.5-EYFP cardiac progenitor reporter mouse model. Adult cardiac fibroblasts infected with all 22 factors yielded some EYFP expressing cells only after doxycycline treatment. The factor pool was subsequently reduced to 11, and eventually to 5 factors. After infection with cardiac factors, EYFP+ cells were first observed 4-6 days after doxycycline treatment. After 3 weeks, EYFP+ cells developed into highly proliferative EYFP+ colonies of cells under optimized culture conditions. The EYFP cells exhibited a high nuclear-to-cytoplasmic ratio and revealed up-regulation of CPC markers including Mesp1, Mef2c, Irx4, Gata6, Tbx5, Tbx 20 as well as downregulation of fibroblast-specific genes such as Fsp1 and Thy1. The EYFP+ cells could be differentiated into various cardiac lineages, including cardiomyocytes (α-MHC), smooth muscle (SM-MHC) and endothelium (CD31). After doxycycline withdrawal cells remained EYFP+, expressed CPC markers, and retained multipotency for over 30 passages, all indicative of stable reprogramming to an iCPC state. iCPCs co-cultured with mESC-derived CMs formed intercellular gap junctions and showed synchronous beating accompanied by spontaneous calcium transients. When injected into the cardiac crescent of E8.5 mouse embryos, iCPCs migrated to the developing heart tube and differentiated into MLC2v and cardiac actin expressing CMs. Similar iCPC reprogramming was achieved using adult lung- and tail tip-derived fibroblasts. In conclusion, we have stably reprogrammed adult fibroblasts from all three germ layers into proliferative and multipotent iCPCs.
ABSTRACT # 9

DNA Binding Specificity Determinants of the Human Pluripotency Transcription Factors Nanog and SOX2

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Sequence-specific DNA binding proteins such as transcription factors are key determinants of cellular state and have been shown to control cell differentiation. Evaluating the specificity profile of DNA binding proteins is a nontrivial challenge that hinders the ability to decipher gene regulatory networks or engineer molecules that act on genomes. The Ansari Lab has developed high-throughput experimental tools that allow the unbiased determination of DNA-binding proteins specificity by interrogating the protein with the entire sequence space of a DNA binding site. The binding preferences for the entire sequence space from 8 to 12 base pairs are visualized as Sequence-Specificity Landscapes (SSLs). Displaying the entire binding spectrum facilitates identifying specificity determinants that are usually hidden and hard to identify by traditional methods. We employed Cognate Site Identification by High-Throughput SELEX to identify specificity determinants of the homeodomain of NANOG, and the high-mobility group box domain of SOX2. Both of these factors are important components of the pluripotency network.
ABSTRACT # 10

Successful Application of Induced Pluripotent Stem Cell Technology for de novo Generation Of Leukemia Stem-Like Cells and Discovery of Novel Leukemia Stem Cell Survival Factor

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A definitive cure for leukemia requires identifying novel therapeutic targets to eradicate LSCs. However, the rarity of LSCs within the pool of malignant cells remains a major limiting factor for their study in humans. Here, we tested the hypothesis that reprogramming leukemia cells to pluripotency and then differentiating them back into blood cells can be used as a novel approach to produce an unlimited number of primitive hematopoietic cells with LSC properties for identifying novel LSC survival factors and drug targets. Recently, we generated transgene-free iPSCs from bone marrow mononuclear cells of a patient in the chronic phase of CML (Hu et al., Blood 2011). By differentiating CML iPSCs back to the blood, we were able to generate primitive hematopoietic cells with typical LSC properties, including lin−CD34−CD45−CD90−CD117−CD45RA−Rhohight ALDHLow primitive hematopoietic phenotype, adhesion defect, increased long-term survival and proliferation, and innate resistance to tyrosine kinase inhibitor imatinib. Using CML iPSC-derived primitive leukemia cells, we discovered a novel factor olfactomedin 4 (OLFM4) that contributes to selective survival and growth of somatic CML LSCs, but not normal primitive hematopoietic cells. Importantly, we found that knockdown of OLFM4 in somatic CML lin−CD34+ cells abrogated their engraftment in NSGw41 mice thereby indicating that OLFM4 is critical for survival of bone marrow repopulating leukemia cells. In conclusion, this is the first study to show the feasibility of using reprogramming technology to develop strategies for targeting LSCs to achieve a cure for leukemia.
Friedman Hub Site 03
**ABSTRACT # 11**

**Src-Activated Runx1 Regulates the Cebpa +37 kb Enhancer in LT-HSC**

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Runx1 is required for development of AGM-derived LT-HSC. The Cebpa gene contains a conserved 450 bp segment at +37 kb that binds Runx1 via four sites. We developed a transgenic mouse in which the +37 kb Cebpa enhancer and promoter direct hCD4 expression. In addition to the majority of GMP, surface hCD4 was evident on 19% of adult marrow or fetal liver LSK; SLAM LT-HSC. To evaluate expression in functional LT-HSC, Ly5.1 hCD4+ or hCD4− marrow was transplanted with equal numbers of Ly5.2 competitors. At 18 wks, hCD4+ cells contributed to 78% and hCD4− cells to only 4% of blood cells, on average, and this striking difference was maintained 18 wks after secondary transplantation. Cebpa-hCD4 expression was also detected in 61% of E11 AGM VEC+CD45+ hematopoietic cells and 11% of VEC+CD45− endothelial cells. VEC+ cells were sorted into hCD4+ and hCD4− subsets, cultured on AGM-derived endothelial cells, and transplanted into syngeneic Ly5.2 recipients; hCD4+ AGM donor cells contributed to a higher percentage of blood cells at 6 and 24 wks. To evaluate the role of Runx1 in regulating transgene expression, Cebpa-hCD4;Runx1(f/f);Mx1-Cre mice were generated and exposed to plpC to induce Runx1 gene deletion; hCD4 expression in LSK;SLAM LT-HSC was markedly reduced. In addition, hCD4+ Ly5.1 cells from these mice were transplanted with equal numbers of Ly5.2 cells. At 16 wks, marrow from donor mice lacking Cre and so retaining Runx1 contributed to 77% and marrow lacking Runx1 to 59% of peripheral blood cells, on average (p = 0.01). Runx1 is phosphorylated by Src on five tyrosines. Runx1(5D), with these residues changed to aspartate to mimic phosphorylation, induced endogenous Cebpa RNA in a myeloid cell line and rescued myelopoiesis in Runx1-deleted marrow cells, whereas Runx1(5F) was ineffective. The Cebpa-hCD4 transgene might facilitate isolation of LT-HSC, and tracking its activity, together with expression of Runx1 (5D) or activation of Src kinases, might facilitate generation of clinically useful LT-HSC from iPSC.
Reprogramming Fidelity Determines the Functional Pluripotency and Lineage Skewing Capacity of Human Induced Pluripotent Stem Cells

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The ideal patient-specific human induced pluripotent stem cell (hiPSC) for clinical use would efficiently generate all tissue types with minimal interline variability or lineage skewing. However, most induced pluripotent stem cells have demonstrated significant limits and variabilities in their multi-lineage differentiation potency. Furthermore, standard hiPSC have been suggested to possess potentially cancer-like epigenetic changes during their reprogramming from adult differentiated cells. Finally, increasing evidence implicate derangements in hiPSC X-chromosome regulation as an obstacle to functional pluripotency. These problems represent a challenge for the full therapeutic realization of hiPSC technology. Genome-wide expression and methylation microarray data on ~50 different human pluripotent stem cell lines was generated in parallel genomics efforts by the Hopkins PCBC Hub, and as part of the PCBC Cell Characterization Core and Bioinformatics Core efforts. Analyses of data from these parallel efforts revealed genetic and epigenetic characteristics that distinguished “stromal-primed” cord blood myeloid progenitor (MP)-derived hiPSC (sp-MP-CBiPSC) derived with extremely high efficiencies. In contrast to hiPSCs derived via standard methods that had highly variable differentiation capacities, sp-MP-CB-iPSC possessed minimal interline variability and a high multi-lineage differentiation potency that lacked lineage skewing. Bioinformatics analyses revealed that sp-MP-CBiPSC largely escaped the acquisition of the typical aberrant hypermethylation signatures routinely observed in standard reprogramming methods, and underwent a more complete erasure of hematopoietic transcriptional and epigenetic donor marks. Finally, sp-MP-CBiPSC were observed to have a greater degree of X-chromosome activation. Taken together, these data demonstrate that efficiently-reprogrammed hiPSC possessed an improved epigenetic quality and more complete reprogramming to functional pluripotency. These data also suggest that future screening and selection of hiPSC quality should be based on identifying hiPSC with fully completed reprogramming to pluripotency, and attempts to “harness” lineage-specific somatic memory to potentiate or augment lineage-directed differentiation should be avoided.

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ABSTRACT # 13

Generation of Clinical-Grade Induced Pluripotent Stem Cells from Human Myeloid Progenitors

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Low reprogramming efficiencies, lack of GMP compliance, and highly variable and limited differentiation into fully functional long-term engraftable lineages are critical challenges for the development of safe translational hiPSC strategies. We, and others have tested the hypothesis that myeloid progenitors (MP) represent an epigenetically “permissive” somatic donor cell type capable of highly efficient reprogramming. We originally reported the bulk reprogramming of human MP with efficiencies of up to 50% using a non-integrating episomal method with stromal priming, and recently reported the enhanced functional multi-lineage performance of human MP-iPSC, including to engraftable vascular progenitors (VP) and functional retinal photoreceptors. Here, we further refine this MP-iPSC reprogramming system using defined cGMP-compliant xeno-free (XF, animal free) and feeder-free (FF) reagents. Human CD34+ CB cells were thawed in RPMI supplemented with XF KnockOut Serum Replacement and expanded and differentiated into CD33+CD45+ MP for 3 days in XF StemSpan hematopoietic expansion medium with the human recombinant proteins FLT3 ligand, TPO and kit-ligand (FTK). Following nucleofection with a single EBNA1-based episomal vector expressing SOX2, OCT4, KLF4 and c-MYC, MP cells were co-cultured on irradiated XF human mesenchymal stromal cells for 3 days. Reprogrammed MPs were transferred onto Synthemax plates in FTK-supplemented StemSpan-XF, and further cultured in FF E8 medium. Distinct hESC-like colonies emerged ~12 days following nucleofection. Single XF-iPSC clones were manually transferred onto human vitronectin-coated plates in E8. All established cell lines expressed pluripotency-associated markers, demonstrated robust differentiation into all three germ layers in NOD/SCID teratoma assay, and retained normal karyotypes. Additionally, XF-MP-iPSC lines efficiently differentiated in vitro into hematovascular, neural, and definitive endodermal lineages at levels comparable to or superior to hESC. We propose that this novel myeloid reprogramming system utilizing an epigenetically “permissive” cell type easily accessible from patient-derived bone marrow, peripheral blood, or HLA-matched CB banks will be an ideal resource for GMP-compliant strategies to generate high quality clinical grade hiPSC for regenerative medicine.
ABSTRACT # 13

Optimized Rewiring of WNT and ERK Pathways is Sufficient for Stable Conversion of Human Pluripotent Stem Cells to a Naïve Ground State of Pluripotency

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Human pluripotent stem cells (hPSC) share developmental, biochemical, and epigenetic commonalities with "primed" rodent post-implantation epiblast stem cells (EpiSC). However, primed EpiSC possess a less primitive and more restricted pluripotency than inner cell mass (ICM)-derived ESC. For example, EpiSC cannot fully contribute to blastocyst chimeras, and possess highly variable directed differentiation potencies. Additionally, mouse ESC can stably convert to ground states of "naïve" pluripotency that requires only small molecule inhibition of GSK3β and MEK/ERK pathways (2i). Recent reports have established that hPSC conversion to naïve pluripotency was inherently unstable, and required supplementation of classical LIF/2i with not only complex inhibitor cocktails that augmented cell survival, but also EpiSC-dependent growth factors (e.g., FGF2/TGFβ). Herein, we demonstrate that optimization of WNT and MEK/ERK modulation was both sufficient and necessary for stably converting both hESC and non-integrated hiPSC to naïve pluripotency. Supplementation of classical LIF/2i medium with one additional WNT/Axin pathway stabilizer (3i) allowed, for the first time to our knowledge, stable conversion of conventional hPSC lines to clonogenic murine ESC-like dome-shaped colonies for >30 passages. Naive hPSCs maintained robust growth kinetics, normal karyotypes, and stably retained TRA antigen expression. Moreover, rewired hPSCs adopted classical murine ESC signaling pathways (e.g., LIF/JAK/STAT; BMP4 signaling), and acquired expressions associated with naïve pluripotency (e.g., increased NANO5, STELLA, NR5A2, KLF2; decreased XIST transcripts). Naive hPSC supported robust tri-lineage teratoma formation, and efficient multi-lineage directed differentiation. Interestingly, although large cohorts of conventional hPSC lines were adapted into 3i, long-term stability was attained only for established hESC and efficiently-reprogrammed myeloid progenitor (MP)-derived hiPSCs, and not for standard fibroblast-derived hiPSCs. These data underscore the importance of exploiting "permissive" somatic donor cell types (e.g., MP) for generating not only high reprogramming efficiencies, but also high-quality hiPSC that can stably convert to naïve pluripotency with only classical WNT-MEK/ERK modulation.
Wu Hub Site 04
ABSTRACT # 14

Transcriptomic Differences between Human Induced Pluripotent Stem Cell Derived Cardiomyocytes and the Adult Human Myocardium

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Cardiomyocytes derived from induced pluripotent cells (hiPSC-CMs) are being used to model cardiac diseases. However, to accurately model these diseases in culture, the transcriptome state of hiPSC-CMs should be highly similar to cardiomyocytes formed in vivo. To compare the transcriptome of in vitro and in vivo derived cardiomyocytes, we performed RNA-seq on hiPSC-CMs and on ventricular and atrial tissue from hearts destined to be transplanted but were rejected due to donor mismatches. RNA-seq performed on hiPSC-CMs fourteen days after small molecule monolayer cardiomyocyte differentiation revealed the transcriptome profile was more similar to the atria. However, the expression of ventricular markers was found after prolonged culture (thirty days after differentiation) suggesting that progressively, a ventricular gene expression profile follows the expression of an atrial gene expression profile in hiPSC-CMs. To determine whether an individual cardiomyocyte exclusively expresses an atrial, ventricular, or a mixed atrial and ventricular gene expression profile, we performed single-cell RNA-seq on hiPSC-CMs (thirty days after differentiation). Clustering the gene expression of each cell identified six populations of cardiomyocytes representing cardiomyocytes at different maturation states expressing either a ventricular (~58% of cells) or atrial-like (~42% of cells) gene expression profile. Pathway enrichment between the atrial and ventricular-like cardiomyocytes was performed to identify distinct signaling pathways between these populations as well as novel gene markers enriched within each population. Future efforts will be placed on engineering hiPSC-CMs to selectively express ventricular, atrial, and mature gene expression profiles.
Novel Codon-Optimized Mini-Intronic Plasmid for Efficient, Inexpensive, and Xeno-Free Induction of Pluripotency

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The development of human induced pluripotent stem cell (iPSC) technology has revolutionized the regenerative medicine field. This technology provides a powerful tool for disease modeling and drug screening approaches. To circumvent the risk of random integration into the host genome caused by retroviruses, non-integrating reprogramming methods have been developed. However, these techniques are relatively inefficient or expensive. The mini-intronic plasmid (MIP) is an alternative, robust transgene expression vector for reprogramming. Here we developed a single plasmid reprogramming system which carries codon-optimized (Co) sequences of the canonical reprogramming factors (Oct4, Klf4, Sox2, and c-Myc) and short hairpin RNA against p53 ("4-in-1 CoMiP"). We have derived human and mouse iPSC lines from fibroblasts by performing a single transfection. Either independently or together with an additional vector encoding for LIN28, NANOG, and GFP, we were also able to reprogram blood-derived peripheral blood mononuclear cells (PBMCs) into iPSCs. Taken together, the CoMiP system offers a new highly efficient, integration-free, easy to use, and inexpensive methodology for reprogramming. Furthermore, the CoMiP construct is color-labeled, free of any antibiotic selection cassette, and independent of the requirement for expression of the Epstein-Barr Virus nuclear antigen (EBNA), which may be beneficial for future applications in regenerative medicine.
ABSTRACT # 16

Molecular Mechanism Underlying Increased Ischemic Damage in the ALDH2*2 Genetic Polymorphism Using a Human iPSC Model System

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About 8% of the human population carry an inactivating point mutation in the cardioprotective enzyme Aldehyde Dehydrogenase 2 (ALDH2), E487K (also denoted as ALDH2*2). This genetic polymorphism is linked to more severe outcomes from ischemic heart damage and increased risk of coronary artery disease (CAD). However, the underlying molecular basis is unknown. Here, we investigated the ALDH2*2 genetic polymorphism and its underlying mechanisms for the first time in a human model system of induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) generated from individuals carrying the most common heterozygous form of the ALDH2*2 genotype. We showed that the ALDH2*2 mutation confers elevated levels of reactive oxygen species (ROS) and toxic aldehydes such as 4HNE, thereby inducing cell cycle arrest and activation of apoptotic signaling pathways, especially during ischemic injury. ALDH2 controls cell survival decisions by modulating oxidative stress levels. This regulatory circuitry was found to be dysfunctional in the loss-of-function ALDH2*2 genotype, causing upregulation of apoptosis in cardiomyocytes following ischemic insult. These results reveal a novel function of the metabolic enzyme ALDH2 in modulation of cell survival decisions. Insight into the molecular mechanisms mediating increased ischemic damage within the ALDH2*2 genotype is important to enable more specific diagnoses and improved risk management of CAD, and may lead to potential patient-specific cardiac therapy.
Pravastatin Reverses Obesity-induced Dysfunction of Induced Pluripotent Stem Cell-Derived Endothelial Cells via a Nitric Oxide-Dependent Mechanism

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Aims: High-fat diet-induced obesity (DIO) is a major contributor to type II diabetes and micro- and macro-vascular complications leading to peripheral vascular disease (PVD). Metabolic abnormalities of induced pluripotent stem cell-derived endothelial cells (iPSC-ECs) from obese individuals could potentially limit their therapeutic efficacy for PVD. The aim of this study was to compare the function of iPSC-ECs from normal and DIO mice using comprehensive in vitro and in vivo assays.

Methods and Results: C57Bl/6 mice were fed with a normal or high-fat diet. iPSCs were generated from tail tip fibroblasts and differentiated into iPSC-ECs using a directed monolayer approach. In vitro functional analysis revealed that iPSC-ECs from DIO mice had significantly decreased capacity to form capillary-like networks, diminished migration, and lower proliferation. Microarray and ELISA confirmed elevated apoptotic, inflammatory and oxidative stress pathways in DIO iPSC-ECs. Following hindlimb ischemia, mice receiving intramuscular (IM) injections of DIO iPSC-ECs had significantly decreased reperfusion compared to mice injected with control healthy iPSC-ECs. Hindlimb sections revealed increased muscle atrophy and presence of inflammatory cells in mice receiving DIO iPSC-ECs. When pravastatin was co-administered to mice receiving DIO iPSC-ECs, a significant increase in reperfusion was observed; however, this beneficial effect was blunted by co-administration of the nitric oxide (NO) synthase inhibitor, Nω-nitro-L-arginine methyl ester (L-NAME).

Conclusion: This is the first study to provide evidence that iPSC-ECs from DIO mice exhibit signs of endothelial dysfunction and had suboptimal efficacy in hindlimb ischemia model. These findings may have important implication for future treatment of PVD using iPSC-ECs in the obese population.
ABSTRACT # 18

Targeted Genetic Engineering of Human Pluripotent Stem Cells Using Transcription Activator–Like Effector Nucleases

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Human induced pluripotent stem cells (iPSCs) represent a unique opportunity for the field of regenerative medicine. To help maximize the potential applications of human iPSCs, we aimed to develop a novel approach to precisely edit the genome of human iPSCs using transcription activator-like effector nucleases (TALENs).

To illustrate the general utility of TALENs for studying signaling pathways and developing functional assays in a common genetic background, we first engineered TALEN vectors to knock out more than 90 individual cardiomyopathy-associated genes. In these experiments, we showed that all TALENS were active and disrupted their target genes at high frequencies. The induction of a DNA double-strand break at the recognition site specifically led to frame shift mutations in the start codon region of these genes, thus ensuring faithful gene knockout events.

We also demonstrated that TALENs can be used to efficiently correct disease-causing mutations in patient-specific iPSCs. Specifically, we were able to successfully correct mutations in the cardiac troponin-T (TNNT2 p. R173W) and β-myosin heavy chain (MYH7 p. R663H) genes that are associated with familial dilated cardiomyopathy (DCM) and hypertrophic cardiomyopathy (HCM), respectively. Importantly, we showed that TALEN-mediated gene correction can result in phenotypic rescue of differentiated iPSC-derived cardiomyocytes (iPSC-CMs).

We evaluated the use of TALENs for targeted integration of a tri-fusion reporter gene into the iPSC genome so that the fate of genome-edited iPSC-CMs can be noninvasively monitored following implantation into living subjects using multimodality reporter gene imaging techniques. The reporter gene specifically encodes for a fusion protein composed of monomeric red fluorescent protein (mRFP), firefly luciferase (FLuc), and herpes simplex virus type 1 thymidine kinase (HSV-1TK), which can be imaged using fluorescence imaging, optical bioluminescence, and PET imaging, respectively.

In conclusion, we have successfully developed powerful TALEN-based genome editing methodologies for both studying cardiovascular disease mechanisms in vitro and safely interrogating stem cell biology in vivo using multimodality molecular imaging techniques.

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ABSTRACT # 19

Identification of Cardiovascular Lineage Descendants at Single Cell Resolution

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Background: The transcriptional profile of cardiac cells derived from murine embryos and from mouse embryonic stem cells (mESCs) has primarily been studied as a cell population. However, the characterization of gene expression in these cells at a single cell level may demonstrate unique variations not appreciated as a pool.

Methods and results: To establish a single cell quantitative PCR platform and perform side-by-side comparison between cardiac progenitors cells (CPCs) and cardiomyocytes (CMs) derived from our previously described Nkx2.5 enhancer-eGFP mESC and mouse embryos, we generated a reference map for single cardiovascular cells through quantifying lineage-defining genes for CPCs, CMs, smooth muscle cells (SMCs), endothelial cells (EDCs), fibroblasts, and mESCs using the Fluidigm microfluidic-enabled multiplex PCR assay. This panel was then applied against day 10.5 embryonic heart single cells to demonstrate its ability to identify chamber-specific CMs, endocardial cells, and fibroblasts. In addition, we compared the gene expression profiles of Nkx2.5 enhancer-eGFP embryo- and mESC-derived CPCs and CMs at different developmental stages and showed that single mESC-derived CM is transcriptionally similar to embryo-derived CM up to the neonatal stage. Furthermore, we show that time-lapse microscopy coupled with single cell expression assay can resolve the identity and the lineage relationship of progenies of single cultured CPC. With this approach, we found differential propensity for mESC-derived CPCs to become SMCs and CMs, whereas embryo-derived CPC to become CMs or EDCs.

Conclusions: Our single cell expression profiling assays demonstrate the transcriptional similarity between mESC and embryo-derived CPC and CM up to the neonatal stage of development as well as differences in the propensity of CPCs to differentiate into cardiovascular cells. Single cell expression analysis appears to be a powerful tool to address the unique behavior of individual embryo- or mESC-derived cardiovascular cells.
ABSTRACT # 20

Cross Talk of Combined Gene and Cell Therapy in Ischemic Heart Disease - Role of Exosomal MicroRNA Transfer

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Despite the promise shown by stem cells for restoration of cardiac function after myocardial infarction, the poor survival of transplanted cells has been a major issue. Hypoxia-inducible factor-1 (HIF1) is a transcription factor that mediates adaptive responses to ischemia. Here, we hypothesize that codelivery of cardiac progenitor cells (CPCs) with a nonviral minicircle plasmid carrying HIF1 (MC-HIF1) into the ischemic myocardium can improve the survival of transplanted CPCs. After myocardial infarction, CPCs were codelivered intramyocardially into adult NOD/SCID mice with saline, MC-green fluorescent protein, or MC-HIF1 versus MC-HIF1 alone (n=10 per group). Bioluminescence imaging demonstrated better survival when CPCs were codelivered with MC-HIF1. Importantly, echocardiography showed mice injected with CPCs+MC-HIF1 had the highest ejection fraction 6 weeks after myocardial infarction (57.1±2.6%, P=0.002) followed by MC-HIF1 alone (48.5±2.6%, P=0.04), with no significant protection for CPCs+MC-green fluorescent protein (44.8±3.3%, P=NS) when compared with saline control (38.7±3.2%). In vitro mechanistic studies confirmed that cardiac endothelial cells produced exosomes that were actively internalized by recipient CPCs. Exosomes purified from endothelial cells overexpressing HIF1 had higher contents of miR-126 and miR-210. These microRNAs activated prosurvival kinases and induced a glycolytic switch in recipient CPCs, giving them increased tolerance when subjected to in vitro hypoxic stress. Inhibiting both of these miRs blocked the protective effects of the exosomes. In summary, HIF1 can be used to modulate the host microenvironment for improving survival of transplanted cells. The exosomal transfer of miRs from host cells to transplanted cells represents a unique mechanism that can be potentially targeted for improving survival of transplanted cells.
ABSTRACT # 21

Cardiac Tissue Slice Transplantation as a Model to Assess Tissue-Engineered Graft Thickness, Survival and Function

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Background: Cell therapies offer the potential to improve cardiac function following myocardial infarction. Although injection of single cell suspensions has proven safe, cell retention and survival rates are low. Tissue-engineered grafts allow cell delivery with minimal initial cell loss and mechanical support to the heart. However, graft performance cannot be easily compared and optimal construct thickness, vascularization, and survival kinetics are unknown.

Methods and Results: Cardiac tissue slices (CTS) were generated by sectioning mouse hearts (n=40) expressing firefly luciferase and green fluorescent protein into slices of defined size and thickness using a vibratome. Bioluminescence imaging (BLI) of CTS transplanted onto hearts of immunodeficient mice demonstrated survival of up to 30% of transplanted cells. Cardiac slice perfusion was re-established within three days through anastomosis of pre-existing vessels with the host vasculature and invasion of vessels from the host. Immunofluorescence showed a peak in cell death three days after transplantation and a gradual decline thereafter. Magnetic resonance imaging (MRI) revealed preservation of contractile function and an improved ejection fraction one month after transplantation of CTS (28±2% CTS vs. 22±2% control; P=0.05). Importantly, this effect was specific to CTS since transplantation of skeletal muscle tissue slices led to faster dilative remodeling and higher animal mortality.

Conclusions: In summary, this is the first study to assess CTS as a benchmark to validate and model tissue engineered graft studies. CTS transplant improved cell survival, established reperfusion, and enhanced cardiac function following myocardial infarction. These findings also confirm that dilative remodeling can be attenuated by topical transplantation of CTS but not skeletal muscle tissue grafts.
ABSTRACT # 22

Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes as a Model for Coxsackievirus B3-Induced Viral Myocarditis

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Viral myocarditis can arise from infection by agents such as the B3 strain of coxsackievirus (CVB3), a member of the enterovirus family. However, procuring primary cardiac tissues by which to study viral myocarditis is risky, invasive, and expensive. Alternatively, human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) can be obtained in unlimited quantities by scaling up and differentiating hiPSCs. In this study, hiPSC-CMs from n=6 patients were infected with CVB3 at multiplicity of infection (MOI) ranging from 5 to 5x10^-6 and subsequently analyzed using luciferase assays, immunofluorescence, calcium imaging, and DNA microarrays. We employed a CVB3-luciferase strain to quantify viral replication on hiPSC-CMs and confirmed that CVB3-Luc elicits maximum cytopathic effect on all infected hiPSC-CMs within 6-8 hours at MOI 5 (p<0.05). At MOI 5x10^-6, cytopathic effect onset is delayed to ~36 hours (p<0.05) because additional CVB3-Luc replication cycles are required for complete hiPSC-CM infection. Immunofluorescence demonstrated that hiPSC-CMs express the coxsackie and adenovirus receptor responsible for viral internalization. CAR is highly upregulated at cell-cell junctions and is also expressed in hiPSCs. Additionally, we utilized bioluminescence imaging and tested the ability of known antivirals such as ribavirin and interferon beta in reducing CVB3-Luc proliferation on hiPSC-CMs. Here we demonstrate the first use of hiPSC-CMs to model CVB3-induced myocarditis. Given the limited treatment options currently available for viral myocarditis and the difficulty of obtaining human cardiac tissue with which to study this disease, this novel platform can be utilized for studying the mechanisms of viral cardiomyopathy and for screening the efficacy of antiviral compounds.
ABSTRACT # 23

**Genome Editing of Isogenic Human Induced Pluripotent Stem Cells Recapitulates Long QT Phenotype for Drug Testing**


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Objectives: To establish an efficient technology to generate human pluripotent stem cells (PSCs)-based disease models with isogenic control.

Background: Human PSCs play an important role in disease modeling and drug testing. However, the current methods are time consuming and lack an isogenic control.

Methods: The ion channel genes KCNQ1 and KCNH2 with dominant negative mutations causing long QT syndrome (LQTS) type-1 and -2, respectively, were stably integrated into a safe harbor AAVS1 locus using zinc finger nuclease (ZFN) technology.

Results: Patch-clamp recording revealed that the edited induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) displayed characteristic LQTS phenotype and significant prolongation of the action-potential duration (APD) compared to the un-edited control cells. Finally, addition of nifedipine (L-type calcium channel blocker) or pinacidil (KATP-channel opener) shortened the APD of iPSC-CMs, confirming the validity of isogenic iPSC lines for drug testing in the future.

Conclusions: Our study demonstrates that PSC-based disease models can be rapidly generated by overexpression of dominant negative gene mutants.
ABSTRACT # 24

Epigenetic Activation of Phosphodiesterase Subtypes Lead to Compromised Beta-Adrenergic Signaling in Induced Pluripotent Stem Cell-Derived Cardiomyocytes from Dilated Cardiomyopathy Patients

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Introduction: Familial dilated cardiomyopathy (DCM) has been modeled by human induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs). However, the mechanisms of compromised signaling transduction and contractile function in DCM iPSC-CMs are still not well understood.

Methods and Results: Beating iPSC-CMs were generated from healthy individuals and DCM patients. RNA-seq and real-time PCR showed strict regulation of the main beta-adrenergic signaling proteins in iPSC-CMs during maturation. Confocal imaging of spontaneous calcium activity and hydrogel-based traction force microscopy (TFM) technology demonstrated beta-adrenergic stimulation induced both inotropic and chronotropic regulation in the contractility of iPSC-CMs. Following extended in vitro maturation of iPSC-CMs, we observed a transition in the beta-adrenergic receptor (beta-AR) subtype dependence from beta-2 AR dominance at early stage (day 30) to beta-1/2 AR co-existence at late stage (day 60). Comparison of the beta-adrenergic responsiveness between iPSC-CMs from DCM patients and their familial controls showed compromised beta-adrenergic signaling in DCM cells. Microarray data and expression profiling indicated up-regulated phosphodiesterases (PDE) 2A, 3A and 5A in DCM iPSC-CMs, which impaired cAMP generation and blunted the beta-adrenergic response. By blocking PDE2A, 3A or 5A, beta-adrenergic signaling reactivity and contractile function in DCM iPSC-CMs were both greatly improved. To further elucidate underlying mechanism of PDE regulation, we conducted chromatin immunoprecipitation (CHIP) assays, which showed significant up-regulation of activation histone marker and down-regulation of repressive histone marker in the PDE promoters during maturation process of DCM iPSC-CMs, which closely recapitulated the epigenetic modulation in the ventricle tissues harvested from DCM patients.

Conclusions: Patient-specific DCM iPSC-CMs recapitulated impaired beta-adrenergic responsiveness and contractility in diseased heart. Studies on iPSC-CM model revealed a novel epigenetic mechanism that underlies PDE subtype specific regulation and signaling deficiency in DCM pathogenesis, which may serve as a new therapeutic target in the future.

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Torok-Storb Hub Site 05
ABSTRACT # 25

Engraftment of ex vivo Expanded, Lentiviral Vector Transduced Hematopoietic Stem/Progenitor Cells in the Dog

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We asked if ex-vivo expanded hematopoietic stem/progenitor cells (HSPC) transduced with a lentiviral vector (LV) could reconstitute hematopoiesis in irradiated dogs. Our goal was to increase the cell dose of autologous genetically modified (GM)-HSPC with a 2-week ex vivo expansion of CD34+ cells. We evaluated the addition of 3 new small molecules: (1) G9a histone lysine methyltransferase inhibitor (UNC0638) and (2) aryl hydrocarbon receptor inhibitor (SR1) and (3) a pyrimidole-indole with stem cell expanding activity in vitro (UM729). The 3 drugs were added throughout the duration of cell culture to improve the ex vivo expansion of CD34+ HSPCs. CD34+ bone marrow cells were isolated from dogs, cultured for 14 days in serum-free media with 4 cytokines (Flt3L, TPO, SCF, IL-6), combined with UNC0638, SR1 and UM729, each at 1 µM. Cells were genetically marked with LV expressing green fluorescence protein (GFP) at day 3 of expansion at a multiplicity of infection of 10. After 14 days of expansion, CD34+ lineage negative cells expanded 12-20-fold and 9%-14% of cells were GFP+. Recipients were conditioned with 9.2 Gray total body irradiation followed by infusion of autologous ex-vivo expanded, transduced HSPCs. Post-grafting immunosuppression consisted of cyclosporine. In the first recipient, on days +7, +14, +21, +28 after GM-HSPC infusion, we detected 3.3%, 5.6%, 5.8%. 2.9% GFP+ granulocytes, respectively. On days +35 and +42, the % GFP+ granulocytes had decreased to 1.5% and 1.2%. GFP+ cells were similarly detected in other blood cell lineages. Engraftment of neutrophils (>500/µL) was on day +26 and platelet recovery was on day +42. Additional transplants in dogs with expanded LV-marked HSPCs are in progress. In summary, CD34+ cells were expanded ex vivo and transduced, and were able to reconstitute hematopoiesis with detection of LV-transduced cells after transplantation in dogs. Long-term follow-up is planned to assess clonal progeny of the LV-marked, expanded HSPCs.
ABSTRACT # 26

Modeling MDS in-vitro Using Patient Derived iPSC

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MDS is a bone marrow failure syndrome, in which clonal ineffective hematopoiesis dominates over time as normal stem cells are inhibited from producing adequate numbers of differentiated progeny. Unlike acute leukemia, clonal dominance does not develop rapidly in MDS, raising the possibility of early therapeutic interventions. While prognosis-associated karyotypic abnormalities can be found in this disease, it is still not clear whether these chromosome abnormalities have anything to do with the cause or progression of MDS but merely reflect the effect of continued genetic instability on the rapidly dividing hematopoietic population. Supporting this hypothesis are the families that carry germline mutations in genes p53, Gata2 and Runx1 whose members are predisposed to developing MDS, but each family member who does develop the disease may have a different marker chromosome. This leads us to speculate there may be a final common pathway for the development of dysplasia independent of specific chromosome abnormalities. We further hypothesized that establishing iPSC lines from MDS patients with predisposing germ-line mutations, and then recapitulating the MDS phenotype during in vitro hematopoietic differentiation and expansion would provide a model for identifying this pathway. To date we have generated iPSC lines from MDS patients with predisposing germ-line mutations, and then characterized by C4). Importantly we have isolated from a single patient iPSC lines with a normal karyotype and lines carrying the distinguishing chromosome marker. We have also generated MDS-like functional phenotypes in vitro. Using these lines to perform a comparative analysis over time we propose to identify the abnormal pathways that participate in the development of the dysplastic phenotype.
**ABSTRACT # 27**

Microvascular Engineering: Recapitulating the Bone Marrow Niche

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In the bone marrow, stromal fibroblasts are found in association with both vascular and hematopoietic cells. Here, we employed an embedded 3D microfluidic vessel system to determine how a third component, normal monocytes, may participate in functional interactions within this system. Microvascular networks are created using soft lithographic technique and injection molding, and endothelial cells self-assemble in the lumen form a responsive and in vivo-like vessel. This system allows us to build a more complete model that mimics marrow matrix and vasculature, allows for 3D geometric cues, controlled hydrodynamic flow and mass transfer, selective multi-cellular compositions, and matrix remodeling. Two functionally distinct marrow-derived fibroblast lines (HS27a and HS5) were embedded in the matrix separately: HS27a expresses stem cell niche-associated proteins, and HS5 secretes copious amounts of growth factors. In the absence of monocytes, both stromal lines reduced endothelial expression of vWF and junctional proteins whereas vessels exposed to HS5 have increased inflammatory cytokines. The functional effect of monocytes was determined by infusing them into the vessels and monitoring their movement. In the presence of HS5 and HS27a, the monocytes adhered to and transmigrated through the endothelium. Adhesion and migration occurred to a significantly lesser extent in the presence of HS5 compared to HS27a. The migrating monocytes made contact with HS27a cells in the matrix, but not HS5 cells. Interactions between monocytes and HS27ain 2D cultures have shown previously to result in the upregulation of proteins associated with the stem cell niche; here, we demonstrate that this also occurs in 3D. However, the relatively stationary non-mitotic behavior of the fibroblasts in 3D cultures differs from that observed in 2D cultures, and more closely approximates their in vivo behavior. Using this platform, it is our intention to reconstruct the cellular interactions that define functionally distinct hematopoietic niches.
Krasnow Hub Site 06
Single Cell Labeling and Clonal Analysis in Mouse Using Cre-loxP Recombination

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Purpose: Vertebrate organogenesis and regeneration are complex processes requiring the coordination of thousands of cells and the proper regulation of essential gene products. Yet in many systems little is known about the behavior of individual cells, such as their patterns of division, movement, and differentiation. Further, it can be difficult to assign precise functions to individual genes since altering expression of key genes often causes global disruption to organ structure. To overcome these problems, we have developed robust, genetically encoded and drug-inducible clonal labeling, mutation and misexpression methods to study development, homeostasis and regeneration in mice.

Methods: Multicolor Cre reporters, such as Rainbow, combined with a cell type specific CreER and limiting doses of tamoxifen, are a robust tool for wild type clonal analysis. By observing labeled clones as organogenesis proceeds patterns of cell proliferation, oriented division, migration and shape change can be directly measured. Controlling the time at which cells are labeled and analyzing the composition of the resulting clones allows the identification and characterization of progenitors of key cell types, and reveals how progenitor behavior changes over time. Creation of definitively labeled mutant or overexpression clones can be achieved using a number of approaches, depending on the specific application. Misexpression using lox-stop-lox overexpression transgenes and the creation of loss of function twinspot clones with the MADM system will be discussed in detail.

Conclusions: Genetically encoded clonal labeling is a powerful technique for revealing the underlying cell dynamics of a system of interest, identification of important progenitor populations and characterization of their changing potential. By overexpressing or mutating key genes in labeled clones within an otherwise wild type organ gene function can be precisely assess gene function in a process of interest while minimize confounding secondary effects.
ABSTRACT # 29

Formation of a Neurosensory Organ by a Novel Mode of Epithelial Cell Migration

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Pulmonary NE cells are a histologically distinct and evolutionarily conserved neurosensory and neurosecretory cell type. While NE cells show increased numbers or altered localization in lung diseases, little is known about their normal development, the control of their proliferation and placement, or their roles in disease outside of their implication as tumor-initiating cells in small cell carcinoma. NE cells in the lung form distinct, innervated clusters called neuroepithelial bodies (NEBs) harboring NE cells that share many morphologic and ultrastructural features of classic neurosecretory cells in other organs. We have elucidated the normal development of NEBs in mice using classic proliferation and cell death markers, and spatially and temporally controlled in vivo genetic cell-marking strategies to sparsely label and track the behavior of individual NE cells. In this presentation, we will discuss recent findings supporting a novel mode of epithelial cell migration to form an intrapulmonary neurosensory structure (NEB).
ABSTRACT # 30

Automated Analysis and Classification of Histological Tissue Features by Multidimensional Microscopic Molecular Profiling

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Characterization of the molecular attributes and spatial arrangements of cells and features within complex human tissues provides a critical basis for understanding processes involved in development and disease. Moreover, the ability to automate steps in the analysis and interpretation of histological images that currently require manual inspection by pathologists could revolutionize medical diagnostics. Toward this end, we developed a new imaging approach called multidimensional microscopic molecular profiling (MMMP) that can measure several independent molecular properties in situ at subcellular resolution for the same tissue specimen, based on repeated cycles of antibody or histochemical staining, imaging, and signal removal. We performed a MMMP analysis on a tissue microarray containing a diverse set of over 100 human tissues using a panel of 15 informative antibody as well as 5 histochemical stains plus DAPI. Large-scale unsupervised analysis of MMMP data, and visualization of the resulting classifications, identified molecular profiles that were associated with functional tissue features. We then directly annotated H&E images from this MMMP series such that canonical histological features of interest (e.g. blood vessels, epithelium) were individually labeled. By integrating MMMP results with image annotation data, we identified molecular signatures associated with specific histological annotations and developed statistical models to automatically classify these features with significant accuracy. Factors that limited our ability to rapidly generate molecular profiles included availability of high-quality antibodies, sample autofluorescence, maintenance of tissue integrity, and turnaround time per cycle. To overcome these technical challenges, we are now pursuing novel strategies for molecular profiling of intact tissues based on RNA in situ hybridization using luminescent lanthanide complexes, which should enable both parallel and serial multiplexing with faster cycling times and negligible autofluorescence.

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Weissman Hub Site 07
ABSTRACT # 31

Interchangeable Fates of Osteogenic and Chondrogenic Progenitors Revealed by Comprehensive Lineage-Mapping of Multipotent Skeletal Stem Cells

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Bone, cartilage, and bone marrow stroma are the primary components of the skeletal system but the origins of these tissues remain undefined. Here we prospectively isolated eight distinct progenitors of bone, cartilage, and stromal tissue and delineated the lineage relationships between them to map bone and cartilage development from a population of highly pure, post-natal skeletal stem cells (BCSsc). The transcriptome of each individual subset of progenitors was investigated to identify unique gene expression programs underlying the intrinsic and extrinsic regulation of BCSsc lineage commitment. These analyses revealed that several types of distinct BCSsc-derived hematopoietic supportive stroma also express factors that can regulate BCSsc expansion and differentiation. We propose that BCSsc generated stroma could play roles in regulating both hematopoietic fates and skeletal fates as cells contributing to both types of niches. We demonstrate that some BCSsc niche factors can be potent inducers of skeletal regeneration, and several specific combinations of recombinant proteins can activate BCSsc genetic programs in situ, even in non-skeletal tissues, resulting in de-novo formation of cartilage or bone and bone marrow stroma.
ABSTRACT # 32

Comprehensive Methylome Map of Human Hematopoietic Stem and Progenitor Cells

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Hematopoiesis is organized as a cellular hierarchy initiated by self-renewing hematopoietic stem cells (HSC) that give rise to multipotent progenitors (MPP), lineage-restricted progenitors, and eventually all the differentiated cells of the blood system. This differentiation hierarchy is dependent upon gene expression changes that result from integration of extracellular signals and epigenetic modifications. Recently, it has been shown that epigenetic change is a driving force for the lineage specific differentiation in murine hematopoiesis. We hypothesized that DNA methylation profiling can discriminate different lineages and identify significant genes that may comprise a signature for each subset in human hematopoietic differentiation. In this study, we examined the DNA methylation status at ~450,000 CpG sites throughout the genome of HSC, MPP, lymphoid-primed multipotential progenitors (L-MPP), common myeloid progenitors (CMP), granulocyte-macrophage progenitors (GMP) and megakaryocyte-erythrocyte progenitors (MEP) from five human bone marrow (BM) donors using the Illumina Infinium HumanMethylation450K BeadChip array. The array design was based on Illumina selection of primers from regions previously described by a consortium of laboratories, and it includes all of the CpG islands and the CpG island shores (regions within 2kb of an island) we previously identified as targets for differential methylation (Ladd-Acosta et al., Nature Genetics, 41: 178-186, 2009). The methylome map showed that the cells are tightly clustered by their lineages. Differential methylation between different lineages is enriched in CpG island ‘shelves’ (regions 2 to 4 kb from islands) and ‘open sea’ (isolated CpG sites in the genome without a specific designation). We identified genes showing progressive DNA methylation change upon differentiation. These genes encode for appropriate lineage-specific developmental functions, including KLF1, ART4 and GATA1, as well as many other previously unknown mediators of lineage specification such as HMHB1 and MIR539. Differential methylation and gene expression of the HSPCs showed a strong inverse correlation, especially at the CpG island shores, and open sea. Global pattern of methylation changes upon differentiation showed loss of methylation on transitions from MPP to CMP, L-MPP to GMP and CMP to MEP. The pattern of epigenetic changes in human hematopoiesis revealed different direction of changes compared to mouse hematopoiesis, indicating intrinsic distinct mechanisms in hematopoietic development in mouse and human. In summary, we have created a comprehensive methylome map of early HSPCs, which should help us to better understand human hematopoiesis. Furthermore, the marked epigenetic clustering of lineages was unexpected and provides strong support for a role in DNA methylation in hematopoietic differentiation, as well as important clues to the dynamics of lineage commitment.
ABSTRACT # 33

Superior Engraftment of Human Normal and Malignant Hematopoietic Cells Using a Novel Humanized Bone Marrow Niche Xenotransplantation Model

Andreas Reinisch and Ravindra Majeti
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Xenotransplantation into immune-compromised mice is currently considered the gold standard to investigate the potency and frequency of human hematopoietic stem and progenitor cells (HSPCs) as well as leukemia-initiating cells (LICs). While these models facilitate engraftment of HSCs and aggressive leukemias, functional in vivo data on more committed progenitor cells and many other hematopoietic malignancies is lacking. This is most likely due to dependency of human hematopoietic cells on interactions with a human bone marrow (BM) microenvironment not provided by recipient mice, therefore emphasizing the need for more suitable mouse models better recapitulating the human BM microenvironment.

Here, we report the development of a novel mouse model bearing a subcutaneously accessible human ossicle formed by in situ differentiation of BM-derived mesenchymal stromal cells. These human ossicles contain a functional humanized BM niche that facilitates engraftment of normal human HSPC and exhibits superior engraftment and expansion of primary AML, including PML-RARA positive APL, a disease that does not engraft in conventional xenotransplantation models.

Human ossicles harboring a fully functional human BM-microenvironment were generated by subcutaneous injection of human BM-derived MSCs. Endochondral ossification of transplanted stromal cells resulted in BM-cavity formation and concomitant invasion of hematopoietic elements. Direct intraossicle injection of human cord blood CD34+ HSPC resulted in robust multi-lineage long-term engraftment including formation of B, T and, NK cells, mature neutrophils, eosinophils, monocytes, mast cells, dendritic cells as well as red blood cell precursors and platelets, as assessed by flow cytometric analysis of aspirates taken directly from the ossicles. Unlike conventional NSG mice, this xenograft model allowed engraftment of phenotypically defined progenitor populations including L-MPP, MLP, CMP, MEP and GMP.

Direct intraossicle transplantation of AML blasts resulted in faster and significantly higher leukemic burden in humanized niches compared to mouse BM and even allowed for engraftment and propagation of PML-RARA-positive APL blasts. By comparing direct intraossicle transplantation with intrafemoral injection into mouse BM, we could detect a 10-250 fold higher LIC-frequency using intraossicle transplantation.

In summary, we report the development of a novel xenotransplantation model bearing a functional human BM niche that substantially improves the engraftment of normal and malignant hematopoietic populations.

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Gene expression profiling using microarray has been limited to profile difference of gene expression at comparison setting since probesets for different genes have different sensitivities. Conventional methods obtain “fold-change” between 2 or more samples, and if a change is not statistically significant, it is scored as “not significant” whether it is expressed or not. Thus scientists could obtain relative difference for limited number of genes and the result is comparison-pair specific.

We tackled this limitation with the hypothesis that if we accumulate a very large number of microarray datasets, meta-analysis could be applied to it to compute dynamic range of each probeset. Furthermore, meta-analysis of data distribution could reveal “actively expressed” range for each probeset with statistical significance. Then mapping individual sample data against those meta-analysis results enables to profile, not relative, but absolute gene expression.

Computer simulations revealed that if size of microarrays exceed 2560, the reproducibility and accuracy of meta-analysis become highly stable. Thus we performed meta-analysis of 11939 Affymetrix mouse 430 2.0 microarray datasets and 25229 Affymetrix human U133 Plus 2.0 microarray datasets downloaded from NIH GEO public repository. Then the strategy is implemented in web-based interface named “Gene Expression Commons” (https://gexc.stanford.edu/). To demonstrate the potential of the Gene Expression Commons, we generated gene expression microarray datasets of 39 hematopoietic populations covering almost the entire quantal stages of mouse hematopoiesis, and have integrated into the system. Gene Expression Commons is designed as an open-platform, thus scientist can upload their own microarray data to profile absolute expression of any gene.
Daley Hub Site 08
Flow-induced Protein Kinase A / CREB Pathway Acts via BMP Signaling to Promote AGM Hematopoiesis

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Fluid shear stress promotes the emergence of hematopoietic stem cells (HSCs) in the aorta-gonad-mesonephros (AGM) of the developing mouse embryo but how mechanical forces are translated into molecular signals for governing HSC emergence is not well understood. We determined that the AGM is enriched for expression of targets of Protein Kinase A (PKA)/CREB, a pathway activated by fluid shear stress. By analyzing CREB genomic occupancy from chromatin-immunoprecipitation sequencing (ChIP-seq) data, we identified the BMP pathway as a potential regulator of CREB. By chemical modulation of the PKA/CREB and BMP pathways in isolated AGM VE-cadherin+ cells from mid-gestation embryos, we demonstrate that PKA/CREB regulates hematopoietic engraftment and clonogenicity of hematopoietic progenitors and is dependent on secreted BMP ligands through the type I BMP receptor. Finally, we observed blunting of this signaling axis using Ncx1-null embryos, which lack a heartbeat and intravascular flow. Taken together, we have identified a novel PKA/CREB-BMP signaling pathway downstream of shear stress that regulates HSC emergence in the AGM. Study of the signaling pathways linking biomechanical stimuli to intracellular events will help elucidate the pathways required to promote HSC formation from pluripotent stem cells for cellular therapy.
ABSTRACT # 36

Notch Signaling Confers Enhanced Lymphoid Potential in Murine ESC/iPSC-derived HSCs

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Transplantation of hematopoietic stem cells (HSCs) from bone marrow, cord blood, or mobilized peripheral blood can be curative for a variety of malignant and genetic blood diseases, but the lack of optimal tissue-matched donors and the immunologic complications that accompany hematopoietic reconstitution limits wider application and causes considerable morbidity and mortality. One strategy for enhanced transplantation outcomes is the creation of autologous HSCs via cellular reprogramming, as it is now feasible to generate induced pluripotent stem cells (iPSCs) from virtually any patient, and to repair gene defects via genome editing. The challenge now is to direct the differentiation of these customized iPSC into engraftable HSC. Our laboratory has previously reported the derivation of murine HSC from ESC (ESC-HSCs) through ectopic expression of HoxB4 and Cdx4 during embryoid body (EB) differentiation, but these ESC-HSC manifest a strong myeloid bias in their differentiation potential. By comparison to the transcriptional profiles of embryonic and fetal HSCs, we found that ESC-HSC closely resemble the definitive HSC that emerge in the developing fetal liver, but are deficient in gene expression programs induced by activation of Notch signaling, which is known to play critical roles in lymphoid development. We thus have employed novel differentiation strategies to enhance Notch pathway activation, including inducible expression of a Notch intra-cellular domain transgene during EB formation, co-culture of the ESC-HSCs on OP9 stroma bearing the Notch ligand DL-1, as well as stromal-free culture in the presence of a tethered Notch ligand. We found that Notch induction within the early hematopoietic specification stage of EB formation diverted cells to a lymphoid-biased fate, whereas titrating the dose of Notch during post-EB in vitro culture enabled the isolation of ESC-HSCs with enhanced in vivo reconstitution of mixed populations of myeloid and lymphoid cells in immune-deficient NSG mice. Reconstituting T lymphocytes displayed a diverse V-beta usage, indicating functional reconstitution activity in these ESC-derived cells. Clonal analysis of sorted lymphoid and myeloid repopulating cells at 16 weeks post-transplantation demonstrated that Notch-enhanced ESC-HSC possess long-term repopulating multi-potentiality. Interestingly, a small subset of engrafted ESC-HSC within bone marrow obtained the Lin− Sca-1$cKit^+$ (ESC-LSK) surface marker expression, phenotypically resembling embryo-derived definitive HSC. Microarray analysis shows that ESC-LSK cluster closer to definitive HSC than do ESC-HSC. We applied CellNet, a network biology algorithm, to expression profiles of ESC-LSK and observed a significant increase of similarity to hematopoietic stem/progenitor cells (HSPC). Lastly, evaluation of NSG mice engrafted with Notch-enhanced ESC-HSC revealed antigen-specific immune-reconstitution and evidence for B and T cell-dependent immunity. Our data suggest that the timing and signal strength of Notch plays a pivotal role in deriving *bona fide* definitive HSC from ESC/iPSC in vitro.
Characterization of Diamond Blackfan Anemia Patient iPS Cells

Diamond Blackfan anemia (DBA) is a rare red cell aplasia that presents in early childhood. Ribosomal protein mutations occur in over 50% of DBA patients. While ribosomes are required in all cells, the major complication of DBA is severely defective erythropoiesis. Isolating patient hematopoietic cells for research is difficult, and the animal models that harbor mutations in ribosomal protein genes have not accurately reproduced the disease. The development of patient-derived induced pluripotent stem (iPS) cells represents a critical in vitro disease model that can be used for understanding disease mechanisms and therapeutic development. We generated iPS cells from DBA patient fibroblasts using retroviral, episomal, and lentiviral reprogramming approaches. We currently have nine lines validated that have a normal karyotype. In order to characterize the blood phenotype of the lines, we generated embryoid bodies (EBs) from two lines derived from a patient with an RPS19 nonsense mutation and one line derived from a patient with an RPL5 nonsense mutation. Isolated hematopoietic progenitors (CD34⁺CD43⁺ cells) from EBs were cultured in erythroid expansion and differentiation media to generate erythroid cells. The three DBA iPS cell lines expanded 5 to 10-fold less than control iPS cell lines. Additionally, the DBA iPS cell lines had decreased production of erythroid cells in two distinct in vitro erythroid differentiation culture systems compared to two control iPS cell lines. At the molecular level, mutations in ribosomal proteins cause p53 activation, which increase downstream transcriptional targets such as p21 mRNA expression. In undifferentiated DBA patient iPS cell lines, there was no change in p21 levels compared to control iPS cell lines. However, in DBA iPS–derived erythroid precursor cells there was a 2 to 5-fold increase in p21 levels compared to control lines, indicating an increase in p53 activity. Taken together, the iPS cell lines derived from DBA patient fibroblast recapitulate the erythroid defect and the p53 activation signature of DBA. These cells will be valuable tools for future studies to understand disease pathology and to screen for new therapies directly in DBA patient cells.
ABSTRACT # 38

Generating Genetically Modified Mice Using CRISPR/Cas9-Mediated Genome Engineering Technology

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CRISPR/Cas9 genome engineering represents an efficient and facile means of editing the genomes of diverse organisms. This method harnesses the adaptive immune defense of bacteria and archaea in which short RNAs guide Cas9 nuclease to specific nucleic acid sequences for degradation. Because the binding of Cas9 is guided by the base-pair complementarities between guide RNA and the target DNA sequence, it is possible to direct Cas9 to any genomic locus by engineering custom single-guide RNAs (sgRNAs). Relative to traditional methods of generating genetically modified mice, CRISPR/Cas9-mediated genome engineering can generate desired strains much more rapidly. Direct co-injection of Cas9 RNA and custom sgRNA into the one-cell mouse embryo circumvents the need for selecting targeted ES cell clones and chimera formation, leading to markedly accelerated generation of mice carrying mutations or alterations in single or multiple genes. Co-injection of a single-stranded or double-stranded DNA oligo can be used to introduce a point mutation, a short fusion tag or a large fragment into an endogenous locus. Reported survival rates after injection are greater than 90%, with efficiency of targeting ranging from 10% to greater than 80% depending on the type of alteration made. Genotyping and confirmation of the desired mutations can be verified by PCR amplification of the sequence flanking the target site, cloning the product into a vector, and validating the alteration by sequencing. Identification of large insertions or deletions should be confirmed by flanking PCR and Southern Blot. Our lab is currently using this technology to generate several strains of mice to tease apart the expression patterns, regulation, and functional redundancy of the stem cell regulators Lin28a and Lin28b. The ease with which this technology can be employed enables rapid generation of powerful tools – both at the animal level and in cell culture— to aid in the in vivo and in vitro study of cellular and organismal phenotypes. Conceptual, practical, and technical aspects and applications will be discussed.
Bernstein Hub Site 09
Induction of Stem Cell Expansion by the Vascular Niche

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Endothelial cells (ECs) play a critical role in the supportive niche of hematopoietic stem cells (HSC) throughout development, from the dorsal aorta of the embryonic AGM (aorta-gonad-mesonephros) region where the first HSC are generated, to the EC sinusoids of the fetal liver where embryonic HSC undergo further maturation and proliferative expansion, and finally in bone marrow where ECs play a role in adult HSC homeostasis. Our prior studies of organ-specific ECs have revealed unique transcriptional profiles suggesting that each organ-specific vascular bed may be endowed with distinctive properties to instruct generation and self-renewal of tissue-resident stem/progenitor cells. Based on these concepts, the overall goal of this Ancillary/Collaborative project is to test the hypothesis that ECs derived from embryonic hematopoietic vascular niches, by supplying unique instructive signals, will significantly increase the specification and expansion of HSC. Consistent with this hypothesis, in our studies to date, we have determined that ECs derived from AGM and fetal liver are capable of inducing long-term engrafting HSC from VE-Cadherin^+ hemogenic precursors derived from pre-engraftment stage murine embryos as early as E9. This includes the population of hemogenic endothelium yet lacking hematopoietic surface markers (VE-Cadherin^+CD45^CD41^), suggesting the vascular niche is able to induce the endothelial to HSC transition in vitro. Furthermore, AGM-ECs have the capacity to significantly increase HSC from E11 AGM-derived CD45^CD45^VE-Cadherin^ hemopoietic cells as determined by limit dilution analysis. Complementary studies have demonstrated that fetal liver and adult bone marrow-derived EC each have the capacity to optimally support the expansion of tissue-resident HSC in vitro. Current efforts are focused on comparing additional EC sources for organ/stage specificity in support of HSC development and expansion in vitro, analyzing the transcriptional profiles of various EC sources to identify organ/stage-specific HSC supportive niche signals, and testing the capacity of these vascular niches to enhance generation of engrafting hematopoietic stem/progenitors cells from embryonic stem cells.
Hematopoietic stem cells (HSCs) develop from a specialized population of endothelial cells known as hemogenic endothelium (HE) during embryogenesis. Sox17 is a critical regulator of HE development both in vivo and during the directed differentiation of embryonic stem cells (ESCs) in vitro. The signaling pathways that regulate Sox17 expression, the transcription co-regulators required for Sox17 function and the transcriptional targets of Sox17 are poorly defined. We applied recently developed methods in quantitative proteomics to further analyze the role of Sox17 in regulating the generation of HE. Specifically, we took advantage of a novel mass spectrometry, the Q Exactive, to perform label-free protein quantification. The Q Exactive combines quadrupole precursor selection with a high-resolution Orbitrap detection. The mass spectrometer’s fast scan rate and simultaneous fill capacity enables the identification of thousands of proteins, maximizing data acquisition at minimal cost. We quantitatively measured Sox17-dependent changes in protein expression during a time course of directed differentiation of murine ESCs using label-free proteomics. We were able to successfully quantify 27,664 peptides corresponding to 3,168 proteins from samples containing as low as 50,000 cells. We are now investigating which proteins are direct transcriptional targets of Sox17. Confirmation of the sensitivity of this proteomic approach was also performed on HE containing cellular fractions isolated from E10.5 and E11.5 murine embryos. Understanding the molecular mechanisms that underlie HE development may facilitate the directed differentiation of HSCs that are capable of long-term engraftment in vivo.
Cooke Hub Site 10
ABSTRACT #41

POU3F2 Regulates Endothelial Cell Differentiation and Vascular Development

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Background: We have developed bi-species heterokaryons [generated by fusion of mouse embryonic stem cells (mESC) and human endothelial cells (hEC)] as a model system for discovery of novel factors required for endothelial lineage. Our preliminary RNAseq data suggests that the determinants of endothelial phenotype in the hEC act on the mESC to recapitulate endothelial ontogeny. In particular, novel transcription factors in endothelial specification were implicated, such as POU domain-containing transcription factor (POU3F2), also called BRN2 or N-Oct3. This study assessed the role of POU3F2 in the endothelial cell differentiation and in the zebrafish vascular development.

Methods and Results: We used mESC to study differentiation towards endothelial lineage. EC differentiation was induced by culture of mESC with growth factors (VEGF, bFGF and BMP4). POU3F2 loss-of-function was induced by lentiviral shRNA in mESCs. FACS was used to analyze cell lineage. Tg (Fli1: eGFP) zebrafish embryos were used to analyze vascular development following caged morpholino (MO) knockdown of POU3F2. Injected caged morpholino was activated at 6 or 24 hour post fertilization by exposure of embryos to UV light. Real Time PCR and Western blotting were used to analyze gene and protein expression respectively. POU3F2 knockdown in mESCs reduced Flk1+CD144+ cell population during differentiation of mESCs. POU3F2 knockdown also reduced endothelial cell markers in mESC derived ECs, including Kdr, Cdh5, Nos3, Tie2 and Lmo2 and reduced EC tube formation in matrigel. In zebrafish embryos, micro-injection of MOs targeting POU3F2 reduced POU3F2 protein at 24 and 48 hpf. This was associated with an embryo phenotype characterized by severe vascular aberrations.

Conclusion: Our heterokaryon studies implicated the transcription factor POU3F2 in endothelial cell development. We validated the role of POU3F2 in the reprogramming of mESC to EC lineage. In addition, we show that POU3F2 is required for normal vascular development in the zebrafish.
ABSTRACT #42

High-Resolution Lineage Mapping of Cells by CyTOF Mass Cytometry

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Single cell studies provide the unique opportunity to elucidate the cellular behavior and molecular control of stem cell fate. Only recently has it become possible to study stem cell behavior and signaling pathways at the single-cell level. Single-cell mass cytometry (CyTOF) is a cutting edge technology at the interface between mass spectrometry and flow cytometry, which allows quantitative, high-throughput analysis of multiple (> 50) biological cell surface, transcription factors and signaling molecules simultaneously in rare populations at the single cell level, by using antibodies labeled with heavy metals (Bendall et al, 2011). We are capitalizing on single-cell mass cytometry to address a highly significant and critical challenge in the field of skeletal cardiac myogenesis, the molecular and functional heterogeneity of muscle stem cells and non-muscle cells within muscle tissues. CyTOF provides an unprecedented opportunity to elucidate the basis for the “defective” stem cell subpopulations and their signaling properties that characterize the progression of muscle diseases and that go awry with aging.
Telomere Extension Using Modified mRNA Encoding TERT

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Telomere extension has been proposed as a means to improve cell culture and tissue engineering, and to treat disease. However, telomere extension by non-viral, non-integrating methods remains inefficient. Here we report that delivery of modified mRNA encoding TERT to human fibroblasts and myoblasts increases telomerase activity transiently (24-48 h) and rapidly extends telomeres, after which telomeres resume shortening. Successive transfections over a four-day period extended telomeres up to 0.9 kb in a cell type-specific manner in fibroblasts and myoblasts and conferred an additional 28 ± 1.5 and 3.4 ± 0.4 population doublings, respectively. Proliferative capacity increased in a dose-dependent manner. Notably, unlike immortalized cells, all treated cell populations eventually stopped increasing in number and exhibited senescence markers to the same extent as untreated cells. This rapid method of extending telomeres and increasing cell proliferative capacity without risk of insertional mutagenesis should have broad utility in disease modeling, drug screening, and regenerative medicine.
ABSTRACT #44

Optimized Plasmid-Based Approach for Generation of High Quality Modified Messenger RNA

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Modified messenger RNAs (mmRNAs), represent a new class of biomimetic polymers with wide applications in the field of regenerative medicine for both research and therapeutic purposes. The RNAcore at Houston Methodist Research Institute has developed an advanced technology for the generation of high quality mmRNAs that is superior to traditional multi-stage production process. We use a plasmid-based approach which allows us to incorporate almost all known RNA, coding and noncoding, sequences by taking advantage of in vivo plasmid expansion to produce longer, error-free RNAs up to 15kb in length. Expression of specific genes can be induced without the concerns (eg. integration of foreign DNA) of other methods such as viral vectors. We have synthesized mmRNAs used in projects relating to reprogramming to pluripotency, transdifferentiation of somatic cells to another somatic cell type (including induction of intermediate progenitors) and cell rejuvenation. For example our colleagues were able to induce both human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) towards pan-hematopoiesis using our mmRNA encoding transcription factors Gata2 and Etv2 (Nature Comm, 2014). In conclusion, the RNAcore has transformed traditional methods of RNA synthesis into an optimized protocol for the generation of high quality modified RNAs which can be used to modulate gene expression. Our mission is to serve the scientific community in producing high quality modified mRNA for any gene of interest for use in advanced regenerative research leading to clinical applications.
Hatzopoulos Hub Site 11
ABSTRACT # 45

GREM2 is a BMP Antagonist that Directs Human iPS Cell Cardiac Differentiation

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Modulation of BMP signaling is known to affect the yield and properties of cardiac cells in development and pluripotent stem cell differentiation. We have previously shown that the secreted BMP antagonist Gremlin 2 (Grem2 or PRDC) plays an important role in cardiac development. Specifically, loss of function studies carried out by our laboratory in zebrafish embryos have shown that proper heart development requires functional Grem2, while overexpression leads to ectopic cardiac tissue formation. Our recent studies showed that in differentiating mouse embryonic stem (ES) cells, Grem2 treatment leads to robust induction of cardiac genes and increases the numbers of cardiomyocytes generated by as much as 100-fold vs. non-treated controls. We further showed that Grem2 treatment induced cardiomyocytes with an atrial-like phenotype. Ongoing work in differentiating human induced pluripotent stem (iPS) cells indicates that GREM2 expression is induced as cardiac progenitor cell colonies form, and continues during cardiac differentiation. Treatment of hiPS cells with Grem2 strongly promotes robust cardiomyogenesis at higher levels than current cardiac induction protocols. The resulting human cardiomyocytes express predominantly atrial genes with suppressed expression of ventricular markers. These results highlight the role of BMP signaling in cardiogenesis and cardiac fate decisions and suggest new strategies for directing differentiation of hiPS cells toward atrial-specific cardiomyocytes for use in disease modeling, drug discovery, and regenerative medicine.
Small molecules that impact cell fate determination and modulate cell differentiation have proven valuable for cellular reprogramming and directed differentiation of pluripotent stem cells toward desired cell types. The impact of small molecules is anticipated to expand to include direct reprogramming as well as de novo progenitor cell mobilization. The Vanderbilt Institute of Chemical Biology (VICB), a trans-institutional initiative between the College of Arts and Sciences and the School of Medicine comprised of over 80 faculty members and four research core facilities, is well equipped to provide seamless integration of chemistry and regenerative biology. The VICB maintains an open-access approach to research, as highlighted by four VICB research core facilities: 1) High-Throughput Screening Core, 2) Antibody and Protein Resource Core, 3) Chemical Synthesis Core, and 4) Small Molecule NMR Core. These four research cores are just a sample of the powerful instrumentation, world-class faculty, outstanding staff and extraordinary facilities that can be found under one roof at Vanderbilt. The VICB’s resources and expertise were instrumental to the first reported use of selective chemical inhibitors of the bone morphogenetic protein (BMP) and Wnt pathways to induce cardiomyogenesis and neurogenesis in pluripotent stem cells. Here, we demonstrate the capability and utility of this Core for medicinal chemistry and bulk synthesis of high-quality small molecule inhibitors of BMP signaling for use in in vitro cardiomyogenesis as well as in vivo signaling modulation in mouse models. The VICB welcomes opportunities to partner with stem cell biologists to help solve important problems and make exciting discoveries in the field.
ABSTRACT # 47

Canonical Wnt Signaling Optimizes Cardiac Tissue Repair by Modulating Cardiomyocyte and Vascular Regeneration

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Wnt signaling pathway regulates the growth and differentiation of cardiac progenitor cells during development and embryonic stem cell differentiation. We have previously shown that canonical Wnt signaling is re-activated in the adult heart after acute myocardial infarction (MI). Wnt activity is induced specifically during the repair phase, suggesting Wnt signaling plays a role in cardiac wound healing and regeneration. To test this hypothesis, we generated a mouse model of increased, cardiac-specific canonical Wnt activity by overexpression of the canonical Wnt ligand Wnt10b, which our analysis identified as strongly induced in cardiomyocytes in the peri-infarct zone.

We found that gain-of-Wnt10b function orchestrated a recovery phenotype characterized by robust neovascularization of the infarct zone, reduced scar size, and improved ventricular function compared to wild-type mice. However, unlike classical pro-angiogenic factors, Wnt10b coordinated both endothelial growth and pericyte recruitment, promoting the formation of large coronary blood vessels. Next to improved neovascularization, histological analysis showed that fibrotic tissue in Wnt10b transgenic mice was replaced by cardiomyocytes, indicating Wnt10b stimulated cardiomyogenesis. Consistent with the adult heart results, we found that Wnt10b strongly expanded the growth of embryonic stem cell-derived cardiomyocytes. Our data suggest Wnt10b-induced canonical Wnt signaling can optimize cardiac tissue repair by enhancing cardiomyocyte and vascular regeneration.
Schneider Hub Site 12
Distinct Molecular Mechanisms Coupling Seaweed and Myomatrix Hydrogels to the Cardiac Genome

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How inanimate biomaterial hydrogels like alginate (Alg), a structurally-simple seaweed-derived polysaccharide biopolymer (generally considered biocompatible and bioinert), and myomatrix (MMx), a multicomponent signal-rich extracellular matrix (ECM) preparation from decellularized pig heart, improve contractile function when implanted in ventricular myocardium is incompletely understood at molecular levels. We report that Alg hydrogel’s mass effect in beating mouse heart produced spatially distributed “expansile” stress, altering tissue architecture and driving an adaptive, topographically restricted myocardial gene expression response mediated by MEF2. MMx hydrogel, on the other hand, quickly recellularized, creating an intramyocardial cell islet that transduced strong hypertrophic growth signals to surrounding cardiomyocytes. These studies provide new molecular mechanistic insights into how Alg and MMx hydrogels differentially regulate myocardial biology and contractile function by transducing biomechanical or cell-mediated signals, respectively, to the cardiac genome. Our results will lead to improved biomaterial-based strategies for heart repair.
ABSTRACT # 49

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeat)/Cas9 (CRISPR Associated Protein 9)-mediated Gene Correction for Disease-causing Mutations

Chengzu Long, John R. McAnally, John M. Shelton, Alex A. Mireault, Rhonda Bassel-Duby, Eric N. Olson

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RNA-guided nuclease-mediated genome editing, based on Type II CRISPR/Cas systems, offers a new approach to alter the genome. Cas9, a nuclease guided by single-guide RNA (sgRNA), binds to a targeted genomic locus next to the protospacer adjacent motif (PAM) and generates a double-strand break (DSB). The DSB is then repaired either by non-homologous end-joining (NHEJ), which leads to insertion/deletion (indel) mutations, or by homology-directed repair (HDR), which requires an exogenous template and can generate a precise modification at a target locus. The benefit of this over other gene therapy techniques is that it can permanently correct the ‘defect’ in a gene rather than just transiently adding a ‘functional’ one.

We used CRISPR/Cas9-mediated genome editing to correct the dystrophin gene (Dmd) mutation in the germline of mdx mice, a model for Duchenne muscular dystrophy (DMD), and then monitored skeletal muscle and heart structure and function. Genome editing produced genetically mosaic animals containing 2 to 100% correction of the Dmd gene. Histological analysis of skeletal muscle and heart tissue from these corrected mice showed absence of the dystrophic muscle phenotype and restoration of dystrophin expression. In addition, the degree of muscle phenotypic rescue in mosaic mice exceeded the efficiency of gene correction, likely reflecting an advantage of the corrected stem cells and their contribution to regenerating muscle.

With rapid technological advances of gene delivery systems and improvements to the CRISPR/Cas9 editing system, this strategy may allow correction of disease-causing mutations in the adult tissue or iPSCs (induced pluripotent stem cells) from patients with genetic diseases, such as sickle-cell anemia, amyotrophic lateral sclerosis and cystic fibrosis.
Prevention of Muscular Dystrophy in Mice by CRISPR (Clustered Regularly Interspaced Short Palindromic Repeat)/Cas9 (CRISPR Associated Protein 9)-mediated Gene Editing

Chengzu Long, John R. McAnally, John M. Shelton, Alex A. Mireault, Rhonda Bassel-Duby, Eric N. Olson

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Duchenne muscular dystrophy (DMD) is an inherited X-linked disease caused by mutations in the gene encoding dystrophin, a protein required for muscle fiber integrity. DMD is characterized by progressive muscle weakness and a shortened lifespan, often along with breathing and heart complications. There is no effective treatment.

RNA-guided nucleases-mediated genome editing, based on Type II CRISPR/Cas systems, offers a new approach to alter the genome. It can precisely remove a mutation in DNA, allowing the DNA repair mechanisms to replace it with a normal copy of the gene. The benefit of this over other gene therapy techniques is that it can permanently correct the ‘defect’ in a gene rather than just transiently adding a ‘functional’ one.

We used CRISPR/Cas9-mediated genome editing to correct the dystrophin gene (Dmd) mutation in the germline of mdx mice, a model for DMD, and then monitored skeletal muscle and heart structure and function. Genome editing produced genetically mosaic animals containing 2 to 100% correction of the Dmd gene. Histological analysis of skeletal muscle and heart from these corrected mice showed absence of the dystrophic muscle phenotype and restoration of dystrophin expression. In addition, the degree of muscle phenotypic rescue in mosaic mice exceeded the efficiency of gene correction, likely reflecting an advantage of the corrected stem cells and their contribution to regenerating muscle.

Our experiments provide proof-of-concept that CRISPR/Cas9-mediated genomic editing can correct a causative germline mutation causing muscular dystrophy in a mouse model and prevent development of several characteristic features of the disease. With rapid technological advances of gene delivery systems and improvements to the CRISPR/Cas9 editing system, this strategy may allow correction of disease-causing mutations in the muscle tissue or iPSCs (induced pluripotent stem cells) from patients with genetic diseases.
ABSTRACT # 51

Myocardial Injury Stimulates Epicardium-Derived Cells to Differentiate into Epicardial Fat via Activation of IGF1R

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There is a strong association between epicardial fat volume and cardiac disease. Although 20% of human heart mass is epicardial fat, the origin and paracrine signaling pathways that regulate its formation and expansion are unclear. Here, we demonstrate using genetic lineage tracing that within the context of myocardial infarction (MI) epicardium-derived cells (EPDCs) differentiate into epicardial fat. Inactivation of the epicardial transcription factor Wt1 significantly reduced EPDC contribution to epicardial fat, providing confirmatory functional data on the role of epicardium in formation of epicardial fat.

To define the paracrine signaling of these differentiation pathways we isolated post-MI EPDCs and used microarrays to identify robustly expressed receptors. We selected for further study 10 candidate receptors with EPDC-selective expression confirmed by immunostaining. Using modified mRNA, we expressed in vivo the cognate ligands for these 10 receptors and tracked the fate of EPDCs using two independent lineage tracing models (WT1<sup>CreERT2/+::Rosa26<sup>fsRFP</sup></sup> and Cre modified mRNA on Rosa26<sup>fsRFP</sup>). We found that IGF-1 modified mRNA given immediately after MI enhanced EPDC differentiate into epicardial fat. Administration of IGF-1 modified mRNA to the left ventricle induce upregulation of adipogenic genes <i>Fabp4</i>, adiponectin, adipasin, and <i>PPARγ</i> at 10 days after MI. We confirmed IGF-1 stimulation of EPDC differentiation to fat using in vitro culture of isolated EPDCs.

In conclusion, we show that that IGF-1 plays a pivotal role after MI in inducing differentiation of EPDCs into epicardial fat. Ongoing experiments are testing the role of EPDC IGF-1 receptor in this process.
Scadden Hub Site 13
Proximity-Based Single Cell Analysis of the Bone Marrow Niche Identifies Interleukin-18 as a Quiescence Regulator of Early Hematopoietic Progenitors

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Background: Niche-derived signals are essential for maintenance and expansion of stem/progenitor cells in the bone marrow. Recently published studies used elegant genetic tools to dissect predominant cellular sources of known HSPC regulators. However, strategies enabling identification of novel niche-derived factors are lacking. Here, we describe an approach which utilizes spatial proximity between osteolineage cells (OLC) and HSPC in the post-transplant bone marrow niche as a guide to niche factor discovery and reveals the role of Interleukin-18 (IL18) as a quiescence regulator of early hematopoietic progenitors.

Results: We established a neonatal bone marrow (BM) transplantation model, in which fluorescently labeled BM-derived LKS CD34-Flk2- HSPCs were transplanted into irradiated newborn col2.3GFP recipients. We harvested GFP-positive OLCs located either in close proximity or further away from transplanted HSPC and compared their transcriptional profile by single cell RNA-Seq. We found that “proximal” OLCs were distinct from their distal counterparts and enriched for known niche-associated secreted factors and those not previously linked to the niche function, including a pro-inflammatory cytokine IL18.

Cell cycle studies in IL18 KO mice revealed that while long-term HSCs were unaffected by the IL18 deletion, early progenitors (ST-HSC, MPP, CLP) were more actively cycling. Following sublethal exposure to 5-fluorouracil, IL18KO animals displayed a 2-fold increase in frequency of LKS cells, lin-kit+ myeloid progenitors and CLPs. Taken together, these results suggest that IL18 controls progenitor quiescence. The effect of IL18 is non cell-autonomous, as transplanted WT LKS cells produce faster early lymphoid and myeloid reconstitution in IL18KO recipients as compared to WT hosts. Notably, we observed reduced 30-day post-transplant mortality (33% versus 72%, p value = 0.05) in IL18KO mice transplanted with a limiting BM dose, i.e. predicted to confer survival to 50% or less in WT animals, indicating that the loss of IL18-mediated progenitor quiescence can be therapeutically exploited.

Conclusion: Our study demonstrates the capability of proximity-based single cell analysis to identify a novel niche factor – IL 18 - which specifically regulates quiescence of early progenitors. Moreover, improved post-transplant survival in the absence of IL18 suggests that pharmacological inhibition of IL18 has the potential of improving clinical outcomes in transplant recipients.
ABSTRACT # 53

Defining Stem and Stromal Cell Couples in Hematopoiesis

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Stem cells are regulated by intrinsic and niche-derived molecular factors. Niche-derived factors have a large translational potential as they allow external control over stem cell trafficking and fate decisions. Nevertheless, the identification of niche-derived factors regulating hematopoietic stem and progenitor cells (HSPCs) still largely relies on the selection of candidate-molecules, limiting the identification of novel factors. In an effort to identify novel factors in an unbiased manner and with higher throughput, we proposed to perform single cell extraction from the bone marrow and molecular profiling based on proximity to HSPCs.

To enable the comparison of molecular profiles based on relative cell location, we have first developed an optical platform for the extraction of BM stem and stromal cells. For cell capture, we use a new, custom-built optical platform that integrates a laser ablation (cutting) beam and an optical tweezer with a confocal/multiphoton microscope. Laser ablation is achieved by tightly focusing an amplified femtosecond laser pulse to create multiphoton ionization and plasma generation at the focus. Each laser pulse removes ~1 μm³ of bone tissue, providing an exquisitely fine cutting action with minimal collateral damage to surrounding tissue. Larger tissue volume can be removed or dissected by the application of repetitive laser pulses. The procedure is carried out under image guidance with multiphoton microscopy and high-precision micromechanical control. We used this system to access the calvarial bone marrow and capture stromal cell according to their position relative to HSPCs. The cells are captured using micromanipulation techniques and prepared for single-cell transcriptome analysis. We expect that this approach will be applicable to multiple cell types, thus enabling a more global and unbiased study of components of the niche based on proximity to HSPCs.
Optical Platform for Intravital Characterization of Bone Marrow Stem and Progenitor Cells


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We have developed a multi-functional optical platform for in vivo cell tracking, sensing, and micromanipulation of the mouse skull bone marrow (BM). Our goal is to provide a 3D view of the cellular organization and the interaction between hematopoietic cells and stromal cells in the BM, as well as the in vivo dynamics during steady state and under stress. The central component of our platform is a custom confocal and multiphoton video-rate laser scanning microscope designed specifically for live animal imaging. Cellular dynamics such as homing, engraftment, and turnover can be monitored with single cell resolution on the time scale ranging from sub-seconds to days and weeks. In addition, the high-speed acquisition makes it possible to locate rare cells in vivo by rapid scanning of a large tissue volume in 3D.

The optical platform has a flexible open architecture:

- By adding a microbeam for targeted photoconversion, the ability to track individual cells can be extended over time and space.
- Integration of a surgical beam enables precise cutting of tissue (including bone) by plasma-mediated femtosecond laser ablation. With this system, bone defects can be generated under two-photon image guidance at specific location and depth for studying tissue regeneration. In addition, microchannels can be drilled through bone for single cell transplantation into the BM. Conversely, microdissection can be performed for cell harvesting directly from the BM.
- Addition of an optical tweezer enables delivery of a single cell into the BM cavity through the microchannel for longitudinal analysis of the transplanted cell and its progeny.
- Implementation of two-photon phosphorescence lifetime microscopy enables in vivo measurement of local oxygen concentration in the BM microenvironment in order to determine the role of hypoxia (low oxygen tension) in maintaining stem cell quiescence.

Additional capabilities can be integrated to meet specific experimental needs of PCBC investigators.
Morrisey Hub Site 14
ABSTRACT # 55

The Epigenetic and Transcriptional Landscape of Mesoderm Progenitor Cells Identifies Novel Regulators That Direct Cardiac versus Hemogenic Endothelial Fate

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Lineage decisions in cardiac and endothelial fate determination are determined very early in development, but the epigenetic and transcriptional mechanisms that establish the identity of these closely related cell types are poorly understood.

Methods: Using hESC directed differentiation, we titrated Activin A and BMP4 doses to generate anterior and posterior mesoderm and used known developmental cues to push these cells into high purity cardiac and endothelial fates. We then used ChIP-seq and RNA-seq, to analyze chromatin dynamics and transcriptional states underlying mesodermal fate specification of hESC-derived cardiac progenitor cells (CPCs), endocardial-like hemogenic endothelial cells (E-HECs), and vascular-like hemogenic endothelial cells (V-HECs).

Results: Chromatin dynamics and gene expression were used alone or in an integrated algorithm to identify novel mesoderm lineage regulators. As opposed to using expression data alone, which identified primarily structural molecules, identification of regulators by integrating expression and chromatin dynamics resulted in enrichment of transcription factors. Each progenitor population showed a unique profile of known and putative novel regulatory molecules involved in fate specification in mesoderm patterning. HOPX, a non-DNA binding homeobox protein with known roles in cardiac maturation, was identified as a novel regulator of both HEC fates. By generating a HOPX reporter hESC line using CRISPR/Cas9, we show that HOPX is transcriptionally activated in both hemogenic endothelial lineages as well as late stages of cardiac lineage development. Expression of HOPX in adult human primary endothelial cells from multiple tissue sources indicates a role for HOPX in human endothelial homeostasis. Lastly, sashimi analysis of RNA-seq data shows splice isoforms that distinguish unique HOPX transcripts in cardiac vs. endothelial fates that are conserved across all stages of development.

Conclusion: We used transcriptional and epigenetic data to understand mechanisms underlying mesoderm fate determination and identified HOPX as a novel regulator of hemogenic endothelium.
ABSTRACT # 56

A MicroRNA Pathway That Promotes Cardiomyocyte Proliferation and Cardiac Regeneration by Inhibiting Hippo Signaling

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In contrast to lower vertebrates, the mammalian heart has limited capacity to regenerate after injury in part due to ineffective reactivation of cardiomyocyte proliferation. While evidence exists for a low level of cardiomyocyte proliferation in the adult heart, it is insufficient to replenish lost cardiomyocytes after injury and re-establish proper heart function. In this study, we show that the microRNA cluster miR302-367 is expressed in early cardiac development. Loss of miR302-367 leads to decreased cardiomyocyte proliferation during development. In contrast, increased miR302-367 expression leads to high level and persistent cardiomyocyte proliferation and ultimately cardiomegaly. miR302-367 functions, in part, by targeting several components of the Hippo signal transduction pathway including Mst1, Lats2, and Mob1b. Postnatal re-expression of miR302-367 leads to reactivation of the cell cycle in cardiomyocytes resulting in reduced scar formation after infarction. However, long-term expression of miR302-367 leads to cardiomyocyte de-differentiation and dysfunction, suggesting that persistent reactivation of the cell cycle in postnatal cardiomyocytes is not desirable. Importantly, this limitation can be overcome by transient systemic application of miR302-367 mimics, leading to increased cardiomyocyte proliferation and mass, decreased fibrosis, and improved function after injury. In conclusion, our data demonstrate the ability of microRNA based therapeutic approaches to promote cardiac repair and regeneration through the transient activation of cardiomyocyte proliferation.
ABSTRACT # 57

Generation of a Lung Stem Cell “Tool Kit” for Derivation of Functional Lung Epithelial Lineages

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To fulfill the promise of regenerative medicine in the lung, it will be necessary to precisely direct the differentiation of stem/progenitors cells into pure populations of mature and functional epithelium characteristic of the adult lung. Recent evidence shows that both mouse and human pluripotent stem cells (hPSCs) can be induced to generate lung epithelium in vitro via recapitulation of the sequence of developmental milestones that occurs during early embryonic lung formation in vivo. However, these methods are still inefficient; the lineages produced are heterogeneous; and little is understood about the pathways that regulate this process. To address these roadblocks, we sought to generate a stem cell “tool kit” of lung epithelial lineage reporter cell lines in hPSCs for development of methods for derivation of pure populations of proximal and distal epithelial lineages. These cell lines include fluorescent protein reporters for multipotent lung endoderm progenitor cells NKX2.1 and Sox2, functionally important lineages of proximal (SCGB3A2) and distal (SFTPC) lung epithelium and dual reporters of NKX2.1/SCGB3A2 and NKX2.1/SFTPC. The Morrisey group has recently identified a Wnt2-expressing cardiopulmonary mesodermal progenitor (CPP) cell population that is able to give rise to most cells of the cardiac inflow tract and pulmonary mesoderm cell lineages in the developing mouse embryo. Based on these studies, we have generated an hPSC Wnt2-GFP reporter cell line that will allow us to establish the presence of human CPPs and they contribute to co-development of the heart and lung tissues. We are employing TALEN and CRISPR genome editing technologies for creating “knock-ins” of the fluorescent reporters at the promoters of the genes of interest. We introduce floxed antibiotic resistance genes downstream of the reporters to facilitate integration at the specified loci, followed by their removal via Cre recombinase mediated excision. These reporter lines will be made available to all PCBC consortium members for studying lung development in vitro.
ABSTRACT # 58

3-Dimensional Imaging: A Tool to Study Stem Cell Development in vivo

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The whole-mount immunofluorescence (IF) imaging technique renders opaque tissues transparent allowing for the visualization of tissue architecture and cellular niches up to a depth of $500\mu$m. Coupled with laser scanning confocal microscopy and imaging software, tissue architecture and niches can be reconstructed in 3D. This technique is utilized to study the development of stem cells in mouse embryos as well as adult tissues. High-resolution IF imaging of thick samples relies on proper antibody penetration via permeabilization with Triton X-100 and sample clearing via a benzyl alcohol/ benzyl benzoate solution (BABB). BABB is a solvent that dissolves lipids thereby reducing light scattering and optimizing laser penetration. Figure 1 demonstrates that this technique can be used to generate 3D reconstructions of complex tissues such as the vasculature of a mouse embryo, or to acquire high-resolution images of single cells within tissues. The whole-mount IF imaging technique is suited for imaging of up to four markers and is broadly applicable to various tissues and cell types.

Figure 1: Vasculature and hematopoietic cells in the liver of an E11.5 mouse fetus that has been immunostained using the whole-mount IF technique. Red, CD31; blue, Runx1; green, Ly6a (Sca-1)-GFP

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Srivastava Hub Site 15
Understanding the Mechanisms of Direct Cardiac Reprogramming

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We previously reported that the introduction of three cardiac developmental transcription factors, Gata4, Mef2c and Tbx5 (GMT) is sufficient to induce mouse fibroblasts to adopt a cardiomyocyte-like phenotype in vitro and in vivo. Although direct reprogramming of fibroblasts to induced cardiomyocyte-like cells (iCMs) is a promising new approach to cardiac muscle generation and heart repair, the underlying molecular mechanisms that orchestrate this process are unknown. Here we track transcriptional changes in gene expression and interrogate direct interactions of GMT with the genome over the course of cardiac reprogramming in vitro. To characterize transcriptional changes in gene expression during reprogramming, GMT were overexpressed in cardiac fibroblasts isolated from transgenic mice containing the cardiac αMHC-GFP reporter. iCMs were isolated by fluorescence activated cell sorting of GFP+ cells at multiple different time points throughout cardiac reprogramming. Whole transcriptome analyses revealed both transient and persistent patterns of gene expression throughout the processes of cardiac reprogramming. To identify direct DNA targets of GMT, we constructed a synthetically tagged GMT retroviral systems that generated iCMs similar to those generated using the non-synthetically tagged retroviral system. Chromatin immunoprecipitation of tagged GMT revealed striking patterns of GMT: DNA occupancy over time. Together, these studies characterize molecular changes that occur during reprogramming and provide a framework for further mechanistic studies.
Isolation of Single-Base Genome-Edited Human iPS Cells without Antibiotic Selection


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The advent of human induced pluripotent stem cells (iPSCs) and site-specific nucleases has revolutionized our ability to engineer isogenic mutant human iPS cell lines that exactly reflect patients’ mutations, which should significantly advance biomedical research. However, traditional methods for genome editing use antibiotic resistance markers, leaving a genetic “scar” such as loxP sites that can affect the resulting phenotype. A major challenge of “scarless” mutagenesis is that isolating the rare targeted iPS clones is difficult without antibiotic selection.

To solve this problem, we combined droplet digital PCR (ddPCR) technology, TaqMan PCR system, and optimized iPSC culture system in 96-well plates to develop Rare Allele Induction and Detection (RAID). This method allows for precise base-by-base genome editing in human iPSCs followed by efficient detection, sib-selection, and isolation of mutant clones. RAID allows us to determine if targeting events occur right after initial transfection, allowing us to rapidly optimize conditions and only attempt cloning from a pool of cells we know are positive. Moreover, because positive cells are enriched before cloning them, RAID reduces hands-on time to isolate a mutant clone. RAID uses a 96-well format for positive cell screening, so mutagenesis can be multiplexed.

Originally RAID was designed for heterozygous point mutagenesis, but we have applied RAID for other mutagenic strategies such as homozygous point mutagenesis and FLAG-tagging. Moreover, RAID serves as an easy assay format to compare activities of different genome editing tools such as TALENs and CRISPR/Cas9 to induce homology-directed repair (HDR).

In conclusion, RAID is a versatile and efficient platform for scarless mutagenesis in iPSCs. RAID also contributes to advancement of genome engineering tools by serving as an effective assay to measure nuclease-induced HDR. We anticipate that our approach will advance scarless genome engineering by allowing many scientists to use human iPSCs to model human genetics, revert disease mutations, and create engineered alleles with unparalleled precision and efficiency.
Garry Hub Site 16
Endoglin Regulates Mesoderm Specification by Modulating Wnt and BMP Signaling

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In mice, the absence of the TGF-β type III receptor endoglin (Eng) results in embryonic lethality, indicating its essential role during embryogenesis. We have showed that induction of Eng (iEng) results in increased hematopoiesis, which happens at the expense of the cardiac lineage. Considering that Wnt signaling is critical for cardiogenesis, we hypothesized that Wnt activation may be involved in Eng-mediated cardiac repression. Consistently, inhibition of the canonical Wnt signaling pathway abolished Eng overexpression effects: cardiac repression and enhanced hematopoiesis. To determine whether Eng controls Wnt activation, we introduced a β-catenin-dependent reporter into the iEng ES cell line. We observed distinct up-regulation of reporter activity as well as the amount of active β-catenin in Eng-induced EBs, showing that Wnt activation is directly regulated by this receptor. We have previously demonstrated that the positive effect of Eng on hematopoiesis occurs through BMP signaling, suggesting a potential common downstream effector that supports Eng-mediated regulation of BMP and Wnt signals to enhance hematopoiesis. SMAD1, a downstream component of the BMP signaling pathway, has been shown to integrate BMP and Wnt/GSK3 signaling at the phosphorylation level during embryogenesis. By generating inducible SMAD1 ES cell lines that carry mutated GSK3 conserved sites, we observed that these cultures display enhanced hematopoiesis, similarly to iEng, confirming the importance of BMP and Wnt modulation in SMAD1 activation during this process. To further elucidate how Eng specifies early mesodermal precursors into either cardiac or hematopoietic cells, we performed studies using the mesodermal reporter brachyury-GFP ES cell line, which revealed that Eng identifies cells with both hematopoietic and cardiac potential, and that Eng controls lineage specification by modulating BMP and Wnt signals. Whole transcriptome analysis from Eng+ sub-fractions was performed to define the detailed gene expression profiles of early hematopoietic and cardiac precursors as well as the potential gene expression signature governed by BMP and Wnt signals. Our study uncovers a novel role of endoglin as a mediator between BMP and Wnt signaling during mesoderm specification, and provides mechanistic insight on the understanding of how hematopoietic and cardiac progenitors are established during embryogenesis.
ABSTRACT # 62

Acquisition of a Quantitative, Stoichiometrically Conserved Ratio-Metric Marker of Maturation Status in Stem Cell-Derived Cardiac Myocytes

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There is no consensus in the stem cell field as to what constitutes the mature cardiac myocyte. Thus, helping formalize a molecular signature for cardiac myocyte maturation would advance the field. In the mammalian heart, inactivation of the "fetal" TNNI gene, TNNI1 (ssTnI), together in temporal concert with its stoichiometric replacement by the adult TNNI gene product, TNNI3 (cTnI), represents a quantifiable ratio-metric maturation signature. We examined the TNNI isoform transition in human iPSC cardiac myocytes (hiPSC-CMs) and found the fetal TNNI signature, even during long-term culture (9.5 months). Rodent stem cell-derived and primary myocytes, however, transitioned to the adult TnI profile. Acute genetic engineering of hiPSC-CMs enabled a rapid conversion toward the mature TnI profile. While there is no single marker to denote the mature cardiac myocyte, we propose that tracking the cTnI:ssTnI protein isoform ratio provides a valuable maturation signature to quantify myocyte maturation status across laboratories.
Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a disorder caused by mutations in desmosomal proteins that lead to abnormalities primarily in right ventricular cardiomyocytes (CM). A significant number of mutations in ARVC patients have been found in Plakoglobin, a gene known to be involved in cell-to-cell communication at intercalated disks. Studying the mechanism of pathology in ARVC has traditionally been limited by the ability to obtain human CM from ARVC patients. Recent development of iPSC technology has paved the way for generation of patient specific pluripotent stem cell lines for in-vitro modeling of ARVC. Pairing iPSC’s with new gene editing technology, such as CRISPR, adds powerful tools for elucidating mechanistic insights as well as the possibility for gene correction. Here we demonstrate the utility of pairing these technologies to generate a model of ARVC for investigating the effects of Plakoglobin mutations on early cardiac development. Using the CRISPR gene editing system, we generated ARVC patient specific mutations in the Plakoglobin gene (Jup) of mouse iPS cell lines derived from first heart field (FHF; Nkx2.5) and second heart field (SHF; isl1) reporter mice. We demonstrate decreases in expression of desmosomal and gap junction genes as well as cell electrophysiological changes in Plakoglobin deficient (Jup -/+ ) induced cardiomyocytes (iCM) that are consistent with abnormalities in seen ARVC cardiomyocytes. In addition, we found that these changes are more prominent in iCM’s derived from SHF derived cells compared to FHF derived cells. We show that this effect is do to differential expression of Plakoglobin isoforms through alternative spicing that preferentially includes the disease mutation in SHF derived cells compared to FHF derived cells. This effect is also seen in adult ARVC cardiomyocytes as well as induced cardiomyocytes derived from patient hiPSC’s. These studies demonstrate the utility of using cutting edge technologies for identification of mechanistic and developmental aspects of disease pathology that have traditionally been difficult to investigate.
ABSTRACT # 64

Inferring Cell Differentiation Pathway from Single-Cell RNA-seq Data

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Background: The RNA-seq experiment at the single cell resolution emerges as a powerful tool to study the complex transcriptional dynamics of cell differentiation. However, two technical challenges make the analysis and explanation of single-cell RNA-seq data difficult. First, the low amounts of mRNA and the high variability of gene expression within individual cells often lead to so-called ‘dropout’ events where expressed genes can be detected in one cell but not in another cell. The commonly used analysis method like Principal Component Analysis (PCA) cannot distinguish silent genes and dropout events (missing values) and often yield the biased results. Second, known gene markers are usually required to identify specific cell types after clustering the single-cell expression profiles. In the case that unknown cell types exist in the heterogeneous population, it is difficult to establish their relationships with the known cell types.

Results: Here, we describe a novel method to handle these two challenges. We use a weighted non-negative matrix factorization (weighted NMF) method to impute the missing values and in the meanwhile decompose the transcriptome into a few metagenes. Lower weight is assigned to the entries with zero observed expression values or genes with high variability across samples. On synthetic data, weighted NMF has significantly better performance on imputing the expression values of the dropout genes than PCA. Then, we found that the entropy of the metagene expression profiles (metagene entropy) can be used as a proxy to measure the potentials of the cell differentiation. In the differentiation process, the progenitors cells and differentiated cells tend to have high and low metagene entropy, respectively. Based on the metagene entropy, we evaluate the distance and directionality among cells and predict the differentiation path by a shortest path algorithm. Importantly, our method requires no prior knowledge of gene markers of specific cell types. We demonstrate that our method can successfully reconstruct the lineage hierarchies of distal lung epithelium and recover the lineage-specific markers without any prior knowledge.

Conclusion: We present a novel method to impute the dropout events in single-cell RNA-seq data and infer the cell differentiation pathway without any information on known lineage markers. This method will enable us to explore the lesser-known differentiation process and unleash the power of single cell RNA-seq technique.
ABSTRACT # 65

Inferring Dynamic Gene Regulatory Networks in Cardiac Differentiation through the Integration of Multi-Dimensional Data

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Background: Decoding the temporal control of gene expression patterns is key to understanding the complex mechanism of developmental decisions during heart development. High-throughput methods have been employed to systematically study the dynamic and coordinated nature of cardiac differentiation at the global level with multiple dimensions. There is pressing need to develop systems way to integrate these data from individual studies and infer the dynamic regulatory networks in an unbiased fashion.

Results: We developed a two-step strategy to integrate data from (1) temporal RNA-seq, (2) temporal histone modifications ChIP-seq, (3) transcription factor (TF) ChIP-seq and (4) gene perturbation, to reconstruct the dynamic network during heart development. First, we trained a logistic regression model to predict the probability (LR score) of any base being bound by 543 TFs with known positional weight matrices. Second, four dimensions of data were combined by time-varying dynamic Bayesian network model to infer the dynamic networks at four developmental stages in mouse: mouse embryonic stem cells (ESC), mesoderm (MES), cardiac progenitors (CP) and cardiomyocytes (CM). Our method not only infers the time-varying networks between different stages of heart development, but also identifies the TF binding sites at either promoter or enhancers of downstream genes.

The LR scores of experimentally verified ESC and heart enhancers were significantly higher than random regions (p < 10^{-100}), suggesting that a high LR score is a reliable indicator for functional TF binding sites. Our network inference model identified a region with elevated LR score approximately -9400 bp upstream of the transcriptional start site of Nkx2-5, which overlapped with a previously reported enhancer region (-9435 to -8922 bp). TFs such as Tead1, Gata4, Msx2, and Tgif1 were predicted to bind to this region and participate in the regulation of Nkx2-5. Our model also predicted the key regulatory networks for the ESC-MES, MES-CP and CP-CM transitions.

Conclusion: We report a novel method to systematically integrate multi-dimensional omics data and reconstruct the gene regulatory networks. This method will allow one to rapidly determine the cis-modules that regulate key genes during cardiac differentiation.
ABSTRACT # 66

Two Platform Technologies to Study Cardiac Specification in Three Dimensional, ECM-Based Microtissues

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The content, composition and distribution of the extracellular matrix (ECM) of the heart changes predictably with normal development suggesting that the ECM plays a role in directing cardiac cell specification. In support of this possibility, all protocols used to induce cardiomyocyte differentiation to date rely on substrates coating with ECM. However direct testing of the influence of ECM on cardiac specification is limited by lack of appropriate in vitro model systems. Here we describe two different technologies to study how content, composition and the distribution of 3D ECM influence stem cell (SC) differentiation to cardiac cell types. Both technologies could serve as a platform for adaption to the study of other cell and tissue types.

First, we developed a 3D hydrogel system to entrap stem cells and ECM of defined amount and type while maintaining consistent stiffness and small molecule transport. To this end, we used native chemical ligation to cross-link poly (ethylene glycol) macromonomers under mild conditions while entrapping ECM proteins (termed ECM composites) and SCs. ECM of composites encompassed SCs and directed the formation of distinct structures dependent on ECM type. Because this platform can harness multiple ECMS in a composite, we recently completed a multifactorial optimization of ECM formulations that best support cardiomyogenic differentiation of encapsulated induced pluripotent stem cells.

Second, we utilized multiphoton excited photochemistry to fabricate 3D ECM constructs from digital image stacks attained using fluorescence microscopy of ECM-labeled embryonic ventricles. The approach is made possible by mapping the brightness in the image directly to cross-linking concentration in the fabricated construct with high fidelity in terms of covered area (>95%) and intensity variation (>85%). The results are promising for variable fibrillar and reticulate structures of cardiac tissue, and allow for the first time the ability to test the role of ECM distribution on SC differentiation at submicron spatial resolution. In the future, scaffolds created in this way may serve as an effective means to deliver SCs or associated progeny to damaged cardiac tissue.
Identification of the Pax3-Interactome in Mesodermal Cells

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Specification of the skeletal myogenic lineage in the developing somite relies on the concerted action of different transcription factors, including Pax3/7, Meox1/2 and six proteins but, so far, a detailed analysis of their functions is still missing. Using differentiating mouse embryonic stem cells, which recapitulate the early stages of embryonic development, we show that only Pax3/7 have the ability to activate the skeletal myogenic program in the developing mesoderm, following a precise time-dependent dynamic. After mass spectrometry analysis of the Pax3-containing complexes from early mesodermal progenitors, we identified several proteins involved in chromatin regulation. Among the candidate cofactors, our preliminary data suggest that Ldb1, a mediator of long-distance chromatin interactions, is required for the proper expression of the well characterized Pax3-target gene Myf5. These findings and ongoing studies aim to evaluate the cooperation of Pax3 with other transcription factors/cofactors to ultimately elucidate the myogenic transcriptional regulation during development.
Parker Hub Site 17
Multiplex Analysis of Gene Expression in Individual Cells

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Messenger RNA (mRNA) is a large family of RNA molecules that convey genetic information for protein synthesis. In addition to having been widely recognized as playing a central role in protein expression and localization, they have also been used as molecular biomarkers. The optical imaging of mRNA in living cells is essential for the real-time identification of cells with distinct genetic profiles and for the temporal tracing of gene expression. Recent technical advances have facilitated live-cell mRNA imaging by employing either nucleic acid or protein-based probes. We have devised a novel technology for the multiplex analysis of gene expression in individual cells (MAGIC). Using a fluorescence resonance energy transfer (FRET) approach by combining fluorescently-labeled antisense RNA probe and double-stranded RNA binding protein, we are able to selectively visualize hybridized probes in single living cells. We demonstrate the feasibility of our approach for live-single cell analyses by visualizing the β-actin mRNA in human embryonic stem cell-derived cardiac myocytes (hESC-CMs) and show that our methodology specifically detects the hybridized probe. Currently, pluripotent stem cell-based approaches for the modeling of human heart disease in vitro are limited by the variable degree of phenotypic maturation of CMs and the inability to distinguish ventricular CMs (vCMs) from other subsets of CMs. Myosin heavy chain α (MHCα) has been suggested to play a key role in cardiac maturation. Using MHCα-specific probes and immunofluorescence staining, we first demonstrate that our technology detects transcripts with a high degree of specificity and sensitivity. We then show that MHCα-positive CMs possess more mature calcium handling properties than MHCα-negative CMs. Myosin light chain 2v (MLC2v) has been shown to be specifically expressed by vCMs. Using a specific probe against MLC2v, we selectively identify vCMs among a heterogeneous population of CMs and again demonstrate a high degree of specificity and sensitivity of our system. In conclusion, we have developed a novel approach for the real-time tracking and phenotypic characterization of single living cells with distinct genetic profile to perform concurrent gene expression and functional analyses. We anticipate our technology to open new avenues for applications where cellular heterogeneity plays an important biological role and importantly to allow for the multiplex functional examination of single living cells.
ABSTRACT # 69

Atypical Protein Kinase C Dependent Asymmetric Cell Division Directs Myocardial Trabeculation

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A hallmark of cardiac development is the formation of myocardial trabeculations exclusively from the luminal surface of the primitive heart tube. Herein, we demonstrate that atypical Protein Kinase C Iota (Prkci) and its interacting partners of the cellular polarity machinery are localized to the luminal side of luminal myocardial cells. Remarkably, these cells undergo polarized cell division with the mitotic spindle oriented perpendicular to the heart’s lumen and this polarization requires a normal composition of the cardiac jelly. Disruption of the polarity complex through cardiac specific deletion of Prkci or its downstream interacting partner NuMA, results in aberrant mitotic spindle alignment, loss of asymmetric cardiomyocyte division, and loss of normal myocardial trabeculation and septation. In vitro, myocardial cells can be induced to spatially polarize and this cell polarization is dependent on atypical Protein Kinase C activity. Finally using a lineage tracing approach, we show that cells in individual developing trabeculations are derived from the same parent cell. Collectively these results layout a new paradigm for cardiac morphogenesis where, in response to inductive signals, Prkci and its downstream partners direct asymmetric cell division of luminal myocardial cells to drive trabeculation and septation in the early developing heart.
ABSTRACT # 70

Rapid and Efficient Generation of Vascular Endothelial and Smooth Muscle Cells from Human Pluripotent Stem Cells

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The use of human pluripotent stem cells for in vitro disease modeling and clinical applications requires protocols that convert these cells into relevant adult cell types. Here, we report the rapid and efficient differentiation of human pluripotent stem cells into vascular endothelia and smooth muscle cells. We found that GSK3 inhibition in combination with BMP4 treatment rapidly committed pluripotent cells to a mesodermal fate and that subsequent exposure to VEGF or PDGF-BB resulted in the differentiation of either endothelial cells or vascular smooth muscle cells, respectively. Of note, both protocols produced mature cells with efficiencies of greater than 80% within six days. Upon further purification to greater than 99% via surface markers, endothelial cells maintained their identity, as assessed by marker gene expression, and showed relevant in vitro and in vivo functionality. Global transcriptional and metabolomic analyses further confirmed that these cells closely resembled their mature in vivo counterparts. These results suggest that the cells could be used to faithfully model human disease.
ABSTRACT # 71

Figment, a Small Protein Encoded by an Annotated Large Noncoding RNA, Regulates Energy Homeostasis in Adipocytes

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Though considered to be noncoding, several short open reading frames (short ORFs) have been identified in a number of long intergenic non-coding RNAs (lincRNAs). Recent findings suggest that short ORF-encoded polypeptides may possess biological function. However, there are conflicting conclusions about the degree to which these encoded proteins function biologically. Here we report the functional characterization of a highly conserved, predicted short ORF within the human transcript LINC00116. Using CRISPR-mediated genome editing of human pluripotent stem cells and mice, we show that this predicted short ORF produces a protein, termed Figment (FGM) that is required for mitochondrial function in adipocytes and thereby regulates whole body energy homeostasis.
ABSTRACT # 72

Efficient Ablation of Clinically Relevant Genes in Human Hematopoietic Cells Using CRISPR/Cas9


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Hematopoietic stem cells (HSCs) are the regenerative unit of hematopoietic system and maintain hematopoiesis through self-renewal and differentiation. Given the fact that HSCs restore hematopoiesis in transplanted recipients, HSCs are ideal cell type for cell-based therapy of hematological disorders, following genetic manipulation. The development of genome editing methodologies have enabled site-specific gene modifications and raised the possibility of using genome editing to treat a broad range of diseases at the genetic level. Hematopoietic cells are at the forefront of such efforts. The recent emergence of the bacterial Type II CRISPR system as an efficient gene-editing tool has given an impetus to the efforts of genome modification for cellular therapy. Though proven extremely efficacious in wide-ranging biological systems, CRISPR/Cas9 mediated genome editing in clinically relevant primary human somatic cells has not been reported. Here, we report the efficient ablation of two clinically relevant genes, B2M and CCR5, in primary human CD4+ T cells and CD34+ hematopoietic stem and progenitor cells (HSPCs). Use of single gRNA led to highly efficient mutagenesis in CD34+ HSPCs but not in CD4+ T cells. A dual gRNA approach improved gene deletion efficacy in both cell types. CCR5-edited HSPCs retained multi-lineage potential including xenotransplantation capacity. Deep sequencing of CRISPR/Cas9 CCR5-edited CD34+ HSPC-derived effector cells revealed robust on-target gene ablation with minimal off-target events for tested gRNAs. These results demonstrate that CRISPR/Cas9 can efficiently ablate genes in human hematopoietic stem and effector cells with minimal off-target mutagenesis, which could have broad applicability for hematopoietic cell-based therapy.
ABSTRACT # 73

Optogenetic Heart-On-A-Chip for Preclinical Cardiotoxicity Screening

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Heart-on-chip (HoC) platforms recapitulate in vitro not only the structural architecture, but also the electro-mechanical coupling of native myocardium. However, electrical field stimulation, the state-of-the-art method to stimulate in vitro cardiac tissue, has technical limitations for HoC applications: electrical pacing generates toxic free radicals due to irreversible Faradaic reaction. Powering our HoC with cardiomyocytes expressing optically-activated ion channels, we removed the need for electrical stimulation and developed powerful platforms for preclinical cardiotoxicity screening.

Using a lentiviral transfection, we genetically engineered neonatal rat ventricular myocytes (NRVMs) with light sensitive ion channels (Channelrhodopsin and Halorhodopsin) under the control of a cardiac troponin T promoter. At 4 days post infection, NRVMs expressed the fluorescent tag associated with the channels. These were functionally integrated in the cell membrane, as demonstrated by patch-clamp recordings, ultimately allowing us control over mechanical contraction, as assessed by the muscular thin film (MTF). We compared gene expression, cytoskeletal architecture, calcium handling properties, and the contractile properties of non-infected and infected cardiac tissues to find that our strategy was minimally invasive.

We integrated optical stimulation techniques into HoC platforms to measure the electrophysiological activation and mechanical contraction in primary and human stem cell derived cardiomyocytes (hiPSC-CMs) during healthy and arrhythmogenic optical stimulations and in the presence of arrhythmic agents. In particular, high spatial resolution of optical stimulation enabled measurement of frequency and dose dependent effects (1:1 coupling, contractility, rate of tension development, spontaneous frequency, speed of calcium wave propagation, and calcium transient duration) to quinidine, verapamil, and lidocaine. We also optically initiated reproducible, reentrant arrhythmias, which were otherwise difficult to study in-vitro and tested the efficacy of pharmacological agents in such conditions.

In conclusion, we introduced optogenetics tools into our HoC platforms to enable multi-parameter phenotypic profiling of drug responses in both NRVM and, importantly, hiPSC-CM tissues. We validated the capability of the platforms for in-vitro preclinical cardiotoxicity assay at the tissue level with higher throughput, content, and specificity than previously possible. We believe that the future integration of disease specific hiPSC-CMs into the platforms will provide a personalized cardiotoxicity assay to measure different susceptibilities of individuals to candidate drugs.
ABSTRACT # 74

Heart-on-a-Chip: An in vitro Model for the Study of Myocardial Function

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The prevalence of cardiovascular disease, along with the limited predictive performance of current pre-clinical drug screening assays has created a need for experimental platforms that mimic the physiology of the human heart. To this end, we designed a chip-based model of healthy and diseased myocardium that provides clinically relevant quantitative readouts. The Heart-on-a-Chip was designed to incorporate muscular thin film cantilevers in a microfluidic chamber with physiologically relevant scaling of fluid volume to facilitate measurements of contractile stress generation by engineered myocardial tissues. To mimic the multi-scale architecture of in vivo muscle tissue, micro-molded hydrogel substrates were incorporated into the design of the chip to provide templates for directing the self-assembly of cardiac myocytes into anisotropic micro-scale tissues and create a micro-environment with comparable stiffness to the native myocardium that facilitates long-term culture. Human induced pluripotent stem cell-derived (iPSC) cardiac myocytes acquired from patients with inherited cardiomyopathies, coupled with the Heart-on-a-Chip platform, represent a powerful tool for studying the structural and functional deficits associated with these diseases in vitro. Furthermore, the Heart-on-a-Chip allows us to evaluate the ability of healthy engineered human iPSC cardiac tissues to exhibit clinically relevant dose responses to standard test compounds, and test the ability of potentially therapeutic compounds to rescue the disease phenotype. Recent studies of Barth syndrome mitochondrial cardiomyopathy using patient-derived iPSC cardiac myocytes demonstrate the versatility of the Heart-on-a-Chip to recapitulate the physiology and pathophysiology of the heart, as well as, evaluate pharmaceutical treatment strategies.
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495-513-9578

Click on the meeting participant’s name in this directory to view their profile picture and statement of expertise from the PCBC website.
Public Transportation from Airports to Stanford University

Caltrain from San Francisco Airport to Palo Alto
Transit: 1 hour 12 mins
Departs from Millbrae Caltrain Station
Take the Bullet toward San Jose (Train 360).
Arrival: At Palo Alto Caltrain Station
  • Travel: 2 Stops
  • Previous Stop: Hillsdale Caltrain Station

Approximately 1.3 mile walk to campus or catch the Marguerite shuttle route SE on the weekend to campus; passes right in front of the Li Ka Shing Conference Center.
Additional resources:
http://transportation.stanford.edu/marguerite

From San Jose Airport to Palo Alto
Transit 1 hour 4 mins
Departs from College Park Caltrain Station
Take the Local toward Gilroy (Train 156).
Arrival: At San Jose Caltrain Station
Transfer to the Bullet at San Jose Caltrain Station.
Departs from San Jose Caltrain Station
Take the Bullet toward San Francisco (Train 371).
Arrival: At Palo Alto Caltrain Station
  • Travel: 2 Stops
  • Previous Stop: Mountain View Caltrain Station (1.74 miles to Hotel)

Approximately 1.3 mile walk to campus or catch the Marguerite shuttle route SE on the weekend to campus, passes right in front of the Li Ka Shing Conference Center
### Nearby Restaurants

<table>
<thead>
<tr>
<th>Restaurant Name</th>
<th>Location</th>
<th>Cuisine</th>
<th>Price</th>
<th>Website</th>
<th>Phone</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>191 Restaurant</strong></td>
<td>Mountain View</td>
<td>American - $$</td>
<td></td>
<td><a href="http://www.191restaurantbar.com">http://www.191restaurantbar.com</a></td>
<td>(650) 961-1491</td>
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<tr>
<td><strong>Agave</strong></td>
<td>Mountain View</td>
<td>Contemporary Mexican - $$</td>
<td></td>
<td><a href="http://www.agaveca.com">http://www.agaveca.com</a></td>
<td>(650) 969-6767</td>
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<tr>
<td><strong>Amber Dhara</strong></td>
<td>Palo Alto</td>
<td>Indian - $$</td>
<td></td>
<td><a href="http://www.amber-india.com">http://www.amber-india.com</a></td>
<td>(650) 329-9644</td>
</tr>
<tr>
<td><strong>Amber India - Mountain View</strong></td>
<td>Mountain View</td>
<td>Indian - $$</td>
<td></td>
<td><a href="http://www.amber-india.com">http://www.amber-india.com</a></td>
<td>(650) 968-7511</td>
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<td><strong>Anatolian Kitchen</strong></td>
<td>Palo Alto</td>
<td>Turkish - $$</td>
<td></td>
<td><a href="http://www.anatoliankitchenpaloalto.com">http://www.anatoliankitchenpaloalto.com</a></td>
<td>(650) 853-9700</td>
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<tr>
<td><strong>Buca di Beppo - Palo Alto</strong></td>
<td>Palo Alto</td>
<td>Italian - $$</td>
<td></td>
<td><a href="http://www.bucadibeppo.com">http://www.bucadibeppo.com</a></td>
<td>(650) 329-0665</td>
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<td><strong>Bistro Vida</strong></td>
<td>Menlo Park</td>
<td>French - $$</td>
<td></td>
<td><a href="http://bistroidamp.com">http://bistroidamp.com</a></td>
<td>(650) 462-1686</td>
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<tr>
<td><strong>Café Pro Bono</strong></td>
<td>Palo Alto</td>
<td>French - $$$</td>
<td></td>
<td><a href="http://bonvivantcafe.net">http://bonvivantcafe.net</a></td>
<td>(650) 485-3228</td>
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<td><strong>Caffe Riace</strong></td>
<td>Palo Alto</td>
<td>Italian - $$$</td>
<td></td>
<td><a href="http://www.cafferiance.com">http://www.cafferiance.com</a></td>
<td>(650) 328-0407</td>
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<tr>
<td><strong>Cascall</strong></td>
<td>Mountain View</td>
<td>Tapas / Small Plates - $$</td>
<td></td>
<td><a href="http://www.cascalrestaurant.com">http://www.cascalrestaurant.com</a></td>
<td>(650) 940-9500</td>
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<td><strong>Cela's Mexican Restaurant</strong></td>
<td>Palo Alto</td>
<td>Mexican - $$</td>
<td></td>
<td><a href="http://www.celiasrestaurants.com">http://www.celiasrestaurants.com</a></td>
<td>(650) 843-0643</td>
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<tr>
<td><strong>Chez TJ</strong></td>
<td>Mountain View</td>
<td>Contemporary French - $$$</td>
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<td><a href="http://www.cheztj.com">http://www.cheztj.com</a></td>
<td>(650) 328-0407</td>
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<tr>
<td><strong>Cucina Venti</strong></td>
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<td>Italian - $$</td>
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<td><a href="http://www.cucinaventi.com">http://www.cucinaventi.com</a></td>
<td>(650) 254-1120</td>
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<td><strong>Cucina Venti</strong></td>
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<td>Italian - $$</td>
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<td>(650) 254-1120</td>
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<td><strong>Cucina Venti</strong></td>
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<td>Italian - $$</td>
<td></td>
<td><a href="http://www.cucinaventi.com">http://www.cucinaventi.com</a></td>
<td>(650) 254-1120</td>
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<tr>
<td><strong>Flea Street Cafe</strong></td>
<td>Menlo Park</td>
<td>Californian - $$</td>
<td></td>
<td><a href="http://www.coolatez.com">http://www.coolatez.com</a></td>
<td>(650) 854-1226</td>
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<tr>
<td><strong>Galata Bistro</strong></td>
<td>Menlo Park</td>
<td>Mediterranean - $$</td>
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<td><a href="http://www.bistrogalata.com">http://www.bistrogalata.com</a></td>
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<tr>
<td><strong>Gravity Bistro and Wine Bar</strong></td>
<td>Palo Alto</td>
<td>Italian - $$</td>
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<td><a href="http://www.gravitywinebar.com">http://www.gravitywinebar.com</a></td>
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<td>Restaurant Name</td>
<td>Location</td>
<td>Cuisine</td>
<td>Price</td>
<td>Dining Style</td>
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<tr>
<td><strong>Il Fornaio - Palo Alto</strong></td>
<td>Palo Alto</td>
<td>Italian</td>
<td>$</td>
<td>Casual Dining</td>
<td><a href="http://ilfornaio.com">http://ilfornaio.com</a></td>
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<tr>
<td><strong>INDO Restaurant &amp; Lounge</strong></td>
<td>Palo Alto</td>
<td>Asian</td>
<td>$</td>
<td>Casual Elegant</td>
<td><a href="http://www.indorestaurant.com">http://www.indorestaurant.com</a></td>
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<tr>
<td><strong>Joya Restaurant &amp; Lounge</strong></td>
<td>Palo Alto</td>
<td>Latin / Spanish</td>
<td>$$$</td>
<td></td>
<td><a href="http://www.joyarestaurant.com">http://www.joyarestaurant.com</a></td>
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<tr>
<td><strong>La Fontaine Restaurant</strong></td>
<td>Mountain View</td>
<td>Italian</td>
<td>$</td>
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<td><a href="http://www.lafontainerestaurant.com">http://www.lafontainerestaurant.com</a></td>
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<tr>
<td><strong>La Strada Ristorante Italiano</strong></td>
<td>Palo Alto</td>
<td>Italian</td>
<td>$</td>
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<td><a href="http://www.lastradapaloalto.com">http://www.lastradapaloalto.com</a></td>
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<tr>
<td><strong>Left Bank Menlo Park</strong></td>
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<td>Provencal</td>
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<td>Casual Dining</td>
<td><a href="http://www.leftbank.com">http://www.leftbank.com</a></td>
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<td><strong>Lure + Till</strong></td>
<td>Palo Alto</td>
<td>Californian</td>
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<tr>
<td><strong>MacArthur Park - Palo Alto</strong></td>
<td>Palo Alto</td>
<td>American</td>
<td>$$$</td>
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<td><a href="http://www.macarthurparkpaloalto.com">http://www.macarthurparkpaloalto.com</a></td>
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<tr>
<td><strong>Madera - Rosewood Hotel Sand Hill</strong></td>
<td>Menlo Park</td>
<td>Contemporary American</td>
<td>$$$</td>
<td>Casual Dining</td>
<td><a href="http://maderasandhill.com">http://maderasandhill.com</a></td>
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<tr>
<td><strong>Max's Opera Cafe of Palo Alto</strong></td>
<td>Palo Alto</td>
<td>American</td>
<td>$</td>
<td>Casual Dining</td>
<td><a href="http://www.maxworld.com">http://www.maxworld.com</a></td>
</tr>
<tr>
<td><strong>Nola - Palo Alto</strong></td>
<td>Palo Alto</td>
<td>Cajun</td>
<td>$</td>
<td></td>
<td><a href="http://www.nolas.com">http://www.nolas.com</a></td>
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<tr>
<td><strong>Pacific Catch Mountain View</strong></td>
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<td>Seafood</td>
<td>$</td>
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<td><a href="http://www.pacificcatch.com">http://www.pacificcatch.com</a></td>
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<tr>
<td><strong>Pampas Palo Alto</strong></td>
<td>Palo Alto</td>
<td>Brazilian Steakhouse</td>
<td>$$$</td>
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<td><a href="http://www.pampaspaloalto.com">http://www.pampaspaloalto.com</a></td>
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<tr>
<td><strong>Park Balluchi</strong></td>
<td>Mountain View</td>
<td>Indian</td>
<td>$</td>
<td>Casual Dining</td>
<td><a href="http://parkballuchiusa.com">http://parkballuchiusa.com</a></td>
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<tr>
<td><strong>Piccolo Ristorante Italiano</strong></td>
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<tr>
<td><strong>Reposado Restaurant</strong></td>
<td>Palo Alto</td>
<td>Mexican</td>
<td>$$$</td>
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<tr>
<td><strong>Sakoon</strong></td>
<td>Mountain View</td>
<td>Indian</td>
<td>$</td>
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<td><a href="http://www.sakoonrestaurant.com">http://www.sakoonrestaurant.com</a></td>
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<tr>
<td><strong>Sam's Chowder House - Palo Alto</strong></td>
<td>Palo Alto</td>
<td>Seafood</td>
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<td><a href="http://www.samschowderhouse.com">http://www.samschowderhouse.com</a></td>
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<tr>
<td>Restaurant Name</td>
<td>City</td>
<td>Cuisine</td>
<td>Price</td>
<td>Website</td>
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</tr>
<tr>
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<td>Scott’s Seafood - Palo Alto</td>
<td>Palo Alto</td>
<td>Californian</td>
<td>$$$</td>
<td><a href="http://www.scottsseafoodpa.com">http://www.scottsseafoodpa.com</a></td>
<td>(650) 323-1555</td>
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<tr>
<td>Scott’s Seafood Mountain View</td>
<td>Mountain View</td>
<td>Seafood</td>
<td>$$$</td>
<td><a href="http://www.scottsseafoodmv.com">http://www.scottsseafoodmv.com</a></td>
<td>(650) 966-8124</td>
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<tr>
<td>Scratch (Mtn View)</td>
<td>Mountain View</td>
<td>American</td>
<td>$$$</td>
<td><a href="http://www.scratchmtnview.com">http://www.scratchmtnview.com</a></td>
<td>(650) 237-3132</td>
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<tr>
<td>Shiock Singapore Kitchen</td>
<td>Menlo Park</td>
<td>Southeast Asian</td>
<td>$$</td>
<td><a href="http://www.shiokkitchen.com">http://www.shiokkitchen.com</a></td>
<td>(650) 838-9448</td>
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<tr>
<td>Spalti</td>
<td>Palo Alto</td>
<td>Italian</td>
<td>$$</td>
<td><a href="http://www.spalti.com">http://www.spalti.com</a></td>
<td>(650) 327-9390</td>
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<tr>
<td>Tai Pan</td>
<td>Palo Alto</td>
<td>Chinese</td>
<td>$$$</td>
<td><a href="http://www.taipanpaloalto.com">http://www.taipanpaloalto.com</a></td>
<td>(650) 329-9168</td>
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<tr>
<td>Tamarine</td>
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<td>Vietnamese</td>
<td>$$$</td>
<td><a href="http://www.tamarinerestaurant.com">http://www.tamarinerestaurant.com</a></td>
<td>(650) 325-8500</td>
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<tr>
<td>Thaiphoon - Palo Alto</td>
<td>Palo Alto</td>
<td>Thai</td>
<td>$$</td>
<td><a href="http://thaiphoonrestaurant.com">http://thaiphoonrestaurant.com</a></td>
<td>(650) 323-7700</td>
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<tr>
<td>Vaso Azzurro</td>
<td>Mountain View</td>
<td>Italian</td>
<td>$$</td>
<td><a href="http://vasoazzurro.com/">http://vasoazzurro.com/</a></td>
<td>(650) 940-1717</td>
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<td>Vero</td>
<td>Palo Alto</td>
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<td><a href="http://www.veroristorante.com">http://www.veroristorante.com</a></td>
<td>(650) 325-8376</td>
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<td>Xanh Restaurant</td>
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<td><a href="http://www.xanhrestaurant.com">http://www.xanhrestaurant.com</a></td>
<td>(650) 964-1888</td>
</tr>
</tbody>
</table>
# Taxi Service

<table>
<thead>
<tr>
<th>Campus Address:</th>
<th>Hotel Address:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Li Ka Shing Center</td>
<td>The Hilton Garden Inn Mountain View</td>
</tr>
<tr>
<td>291 Campus Drive West</td>
<td>840 E. El Camino Real</td>
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</table>

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<th>Stanford Yellow Taxi Cab</th>
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</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Yellow Town Taxi Cab</th>
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<tr>
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</tbody>
</table>
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- Stanford Cardiovascular Institute
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Index

The index contains a list of submitted abstracts for the Plenary Sessions, Breakout Sessions, and Technology Fair in alphabetical order by the last name of the first author.

Each abstract is identified as BO=Breakout Session (total of 4 breakout sessions), P=Plenary Session, and/or T=Technology Fair. Meeting booklet page numbers on which the abstract is found are provided for easy reference.
<table>
<thead>
<tr>
<th>Last Name</th>
<th>First Name</th>
<th>Abstract Title</th>
<th>Session</th>
<th>Page #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atmanli</td>
<td>Ayhan</td>
<td>Multiplex Analysis of Gene Expression in Individual Cells</td>
<td>BO – 4</td>
<td>100</td>
</tr>
<tr>
<td>Baik</td>
<td>June</td>
<td>Endoglin Regulates Mesoderm Specification by Modulating Wnt and BMP Signaling</td>
<td>BO – 1</td>
<td>92</td>
</tr>
<tr>
<td>Bedada</td>
<td>Fikru</td>
<td>Acquisition of a Quantitative, Stoichiometrically Conserved Ratio-Metric Marker of Maturation Status in Stem Cell-Derived Cardiac Myocytes</td>
<td>BO – 4</td>
<td>93</td>
</tr>
<tr>
<td>Berry</td>
<td>Emily</td>
<td>Understanding the Mechanisms of Direct Cardiac Reprogramming</td>
<td>BO – 2</td>
<td>89</td>
</tr>
<tr>
<td>Bylund</td>
<td>Jeffery</td>
<td>GREM2 is a BMP Antagonist that Directs Human iPSC Cell Cardiac Differentiation</td>
<td>P</td>
<td>71</td>
</tr>
<tr>
<td>Cardenas</td>
<td>Fabian</td>
<td>A Doxycycline-Inducible System for Gene Correction of Patient-Derived Induced Pluripotent Stem Cells</td>
<td>T</td>
<td>16</td>
</tr>
<tr>
<td>Chan</td>
<td>Charles</td>
<td>Interchangeable Fates of Osteogenic and Chondrogenic Progenitors Revealed by Comprehensive Lineage-Mapping of Multipotent Skeletal Stem Cells</td>
<td>P</td>
<td>53</td>
</tr>
<tr>
<td>Churko</td>
<td>Jared</td>
<td>Transcriptomic Differences between Human Induced Pluripotent Stem Cell Derived Cardiomyocytes and the Adult Human Myocardium</td>
<td>P</td>
<td>33</td>
</tr>
<tr>
<td>Dai</td>
<td>Bo</td>
<td>Comprehensive Methylome Map of Human Hematopoietic Stem and Progenitor Cells</td>
<td>BO – 4</td>
<td>54</td>
</tr>
<tr>
<td>Daughters</td>
<td>Randy</td>
<td>Differential Affects of Plakoglobin Isoform Expression in iPSC Models of Arrhythmogenic Right Ventricular Cardiomyopathy</td>
<td>T</td>
<td>94</td>
</tr>
<tr>
<td>Diecke</td>
<td>Sebastian</td>
<td>Novel Codon-Optimized Mini-Intronic Plasmid for Efficient, Inexpensive, and Xeno-Free Induction of Pluripotency</td>
<td>BO – 3</td>
<td>34</td>
</tr>
<tr>
<td>Domian</td>
<td>Ibrahim</td>
<td>Atypical Protein Kinase C Dependent Asymmetric Cell Division Directs Myocardial Trabeculation</td>
<td>BO – 2</td>
<td>101</td>
</tr>
<tr>
<td>Ebert</td>
<td>Antje</td>
<td>Molecular Mechanism Underlying Increased Ischemic Damage in the ALDH2*2 Genetic Polymorphism Using a Human iPSC Model System</td>
<td>BO – 3</td>
<td>35</td>
</tr>
<tr>
<td>Eguchi</td>
<td>Asuka</td>
<td>Artificial Transcription Factors to Control Cell Fate Choices</td>
<td>T</td>
<td>22</td>
</tr>
<tr>
<td>Last Name</td>
<td>First Name</td>
<td>Abstract Title</td>
<td>Session</td>
<td>Page #</td>
</tr>
<tr>
<td>-----------</td>
<td>------------</td>
<td>----------------</td>
<td>---------</td>
<td>--------</td>
</tr>
<tr>
<td>Erwin</td>
<td>Graham</td>
<td>Precision-Targeted Small Molecule Genome Readers and Epigenetic Remodelers to Regulate Transcriptional Networks</td>
<td>BO – 1</td>
<td>23</td>
</tr>
<tr>
<td>Georges</td>
<td>George</td>
<td>Engraftment of ex vivo Expanded, Lentiviral Vector Transduced Hematopoietic Stem/Progenitor Cells in the Dog</td>
<td>BO – 1</td>
<td>45</td>
</tr>
<tr>
<td>Goetsch</td>
<td>Sean</td>
<td>Distinct Molecular Mechanisms Coupling Seaweed and Myomatrix Hydrogels to the Cardiac Genome</td>
<td>BO – 3</td>
<td>75</td>
</tr>
<tr>
<td>Gong</td>
<td>Wuming</td>
<td>Inferring Dynamic Gene Regulatory Networks in Cardiac Differentiation through the Integration of Multi-Dimensional Data</td>
<td>T</td>
<td>96</td>
</tr>
<tr>
<td>Gong</td>
<td>Wuming</td>
<td>Inferring Cell Differentiation Pathway from Single-Cell RNA-seq Data</td>
<td>T</td>
<td>95</td>
</tr>
<tr>
<td>Gu</td>
<td>Mingxia</td>
<td>Pravastatin Reverses Obesity-induced Dysfunction of Induced Pluripotent Stem Cell-Derived Endothelial Cells via a Nitric Oxide-Dependent Mechanism</td>
<td>BO – 2</td>
<td>36</td>
</tr>
<tr>
<td>Guo</td>
<td>Hong</td>
<td>Src-Activated Runx1 Regulates the Cebpa +37 kb Enhancer in LT-HSC</td>
<td>BO – 2</td>
<td>28</td>
</tr>
<tr>
<td>Gupta</td>
<td>Rajat</td>
<td>Rapid and Efficient Generation of Vascular Endothelial and Smooth Muscle Cells from Human Pluripotent Stem Cells</td>
<td>BO – 3</td>
<td>102</td>
</tr>
<tr>
<td>Hadland</td>
<td>Brandon</td>
<td>Induction of Stem Cell Expansion by the Vascular Niche</td>
<td>BO – 2</td>
<td>63</td>
</tr>
<tr>
<td>Hayes</td>
<td>Brian</td>
<td>Modeling MDS in-vitro Using Patient Derived iPSC</td>
<td>BO – 3</td>
<td>46</td>
</tr>
<tr>
<td>Hong</td>
<td>Charles</td>
<td>Research Cores at the Vanderbilt Institute of Chemical Biology (VICB)</td>
<td>T</td>
<td>72</td>
</tr>
<tr>
<td>Hudak</td>
<td>Carolyn</td>
<td>Figment, a Small Protein Encoded by an Annotated Large Noncoding RNA, Regulates Energy Homeostasis in Adipocytes</td>
<td>BO – 1</td>
<td>103</td>
</tr>
<tr>
<td>Huo</td>
<td>Jeffrey</td>
<td>Reprogramming Fidelity Determines the Functional Pluripotency and Lineage Skewing Capacity of Human Induced Pluripotent Stem Cells</td>
<td>BO – 4</td>
<td>29</td>
</tr>
<tr>
<td>Jarocha</td>
<td>Danuta</td>
<td>Comparative Analysis of Infused “Static”, ex vivo-generated Platelets vs. Infused Megakaryocytes-generated Platelets: A Cautionary Tale</td>
<td>BO – 4</td>
<td>17</td>
</tr>
<tr>
<td>Last Name</td>
<td>First Name</td>
<td>Abstract Title</td>
<td>Session</td>
<td>Page #</td>
</tr>
<tr>
<td>-----------</td>
<td>------------</td>
<td>----------------</td>
<td>---------</td>
<td>--------</td>
</tr>
<tr>
<td>Karakikes</td>
<td>Ioannis</td>
<td>Targeted Genetic Engineering of Human Pluripotent Stem Cells Using Transcription Activator–Like Effector Nucleases</td>
<td>T</td>
<td>37</td>
</tr>
<tr>
<td>Kim</td>
<td>Peter Geon</td>
<td>Flow-induced Protein Kinase A / CREB Pathway Acts via BMP Signaling to Promote AGM Hematopoiesis</td>
<td>P</td>
<td>58</td>
</tr>
<tr>
<td>Kotha</td>
<td>Surya</td>
<td>Microvascular Engineering: Recapitulating the Bone Marrow Niche</td>
<td>T</td>
<td>47</td>
</tr>
<tr>
<td>Kumar</td>
<td>Maya</td>
<td>Single Cell Labeling and Clonal Analysis in Mouse Using Cre-loxP Recombination</td>
<td>T</td>
<td>49</td>
</tr>
<tr>
<td>Kuo</td>
<td>Christin</td>
<td>Formation of a Neurosensory Organ by a Novel Mode of Epithelial Cell Migration</td>
<td>BO – 1</td>
<td>50</td>
</tr>
<tr>
<td>Lalit</td>
<td>Pratik</td>
<td>Lineage Reprogramming Of Mouse Fibroblasts to Proliferative and Multipotent Induced Cardiac Progenitor Cells by Defined Factors</td>
<td>P</td>
<td>24</td>
</tr>
<tr>
<td>Li</td>
<td>Guang</td>
<td>Identification of Cardiovascular Lineage Descendants at Single Cell Resolution</td>
<td>BO – 2</td>
<td>38</td>
</tr>
<tr>
<td>Long</td>
<td>Chengzu</td>
<td>Prevention of Muscular Dystrophy in Mice by CRISPR (Clustered Regularly Interspaced Short Palindromic Repeat)/Cas9 (CRISPR Associated Protein 9)-mediated Gene Editing</td>
<td>P</td>
<td>77</td>
</tr>
<tr>
<td>Long</td>
<td>Chengzu</td>
<td>CRISPR (Clustered Regularly Interspaced Short Palindromic Repeat)/Cas9 (CRISPR Associated Protein 9)-mediated Gene Correction for Disease-causing Mutations</td>
<td>T</td>
<td>76</td>
</tr>
<tr>
<td>Lu</td>
<td>Yi-Fen</td>
<td>Notch Signaling Confers Enhanced Lymphoid Potential in Murine ESC/iPSC-derived HSCs</td>
<td>BO – 2</td>
<td>59</td>
</tr>
<tr>
<td>Macari</td>
<td>Elizabeth</td>
<td>Characterization of Diamond Blackfan Anemia Patient iPSC Cells</td>
<td>BO – 3</td>
<td>60</td>
</tr>
<tr>
<td>Magli</td>
<td>Alessandro</td>
<td>Identification of the Pax3-Interactome in Mesodermal Cells</td>
<td>BO – 2</td>
<td>98</td>
</tr>
<tr>
<td>Mandal</td>
<td>Pankaj</td>
<td>Efficient Ablation of Clinically Relevant Genes in Human Hematopoietic Cells Using CRISPR/Cas9</td>
<td>BO – 1</td>
<td>104</td>
</tr>
<tr>
<td>Matrone</td>
<td>Gianfranco</td>
<td>POU3F2 Regulates Endothelial Cell Differentiation and Vascular Development</td>
<td>P</td>
<td>66</td>
</tr>
<tr>
<td>Miyaoka</td>
<td>Yuichiro</td>
<td>Isolation of Single-Base Genome-Edited Human iPSC Cells without Antibiotic Selection</td>
<td>T</td>
<td>90</td>
</tr>
<tr>
<td>Last Name</td>
<td>First Name</td>
<td>Abstract Title</td>
<td>Session</td>
<td>Page #</td>
</tr>
<tr>
<td>-----------</td>
<td>------------</td>
<td>---------------------------------------------------------------------------------</td>
<td>---------</td>
<td>--------</td>
</tr>
<tr>
<td>Noh</td>
<td>Ji-Yoon</td>
<td>Inducible GATA1 Suppression as a Novel Strategy to Expand Physiologic Megakaryocyte Production</td>
<td>P</td>
<td>18</td>
</tr>
<tr>
<td>Ogle</td>
<td>Brenda</td>
<td>Two Platform Technologies to Study Cardiac Specification in Three Dimensional, ECM-Based Microtissues</td>
<td>T</td>
<td>97</td>
</tr>
<tr>
<td>Ong</td>
<td>Sang-Ging</td>
<td>Cross Talk of Combined Gene and Cell Therapy in Ischemic Heart Disease - Role of Exosomal MicroRNA Transfer</td>
<td>BO – 1</td>
<td>39</td>
</tr>
<tr>
<td>Paik</td>
<td>David</td>
<td>Canonical Wnt Signaling Optimizes Cardiac Tissue Repair by Modulating Cardiomyocyte and Vascular Regeneration</td>
<td>BO – 4</td>
<td>73</td>
</tr>
<tr>
<td>Palpant</td>
<td>Nathan</td>
<td>The Epigenetic and Transcriptional Landscape of Mesoderm Progenitor Cells Identifies Novel Regulators That Direct Cardiac versus Hemogenic Endothelial Fate</td>
<td>P</td>
<td>84</td>
</tr>
<tr>
<td>Park</td>
<td>Sung-Jin</td>
<td>Optogenetic Heart-On-A-Chip for Preclinical Cardiotoxicity Screening</td>
<td>BO – 2</td>
<td>105</td>
</tr>
<tr>
<td>Park</td>
<td>Tea Soon</td>
<td>Generation of Clinical-Grade Induced Pluripotent Stem Cells from Human Myeloid Progenitors</td>
<td>T</td>
<td>30</td>
</tr>
<tr>
<td>Porpiglia</td>
<td>Ermelinda</td>
<td>High-Resolution Lineage Mapping of Cells by CyTOF Mass Cytometry</td>
<td>BO – 2  &amp; T</td>
<td>67</td>
</tr>
<tr>
<td>Pu</td>
<td>William</td>
<td>Myocardial Injury Stimulates Epicardium-Derived Cells to Differentiate into Epicardial Fat via Activation of IGF1R</td>
<td>BO – 4</td>
<td>78</td>
</tr>
<tr>
<td>Ramunas</td>
<td>John</td>
<td>Telomere Extension Using Modified mRNA Encoding TERT</td>
<td>T</td>
<td>68</td>
</tr>
<tr>
<td>Reinisch</td>
<td>Andreas</td>
<td>Superior Engraftment of Human Normal and Malignant Hematopoietic Cells Using a Novel Humanized Bone Marrow Niche Xenotransplantation Model</td>
<td>BO – 1</td>
<td>55</td>
</tr>
<tr>
<td>Riegler</td>
<td>Johannes</td>
<td>Cardiac Tissue Slice Transplantation as a Model to Assess Tissue-Engineered Graft Thickness, Survival and Function</td>
<td>BO – 4</td>
<td>40</td>
</tr>
<tr>
<td>Riordan</td>
<td>Daniel</td>
<td>Automated Analysis and Classification of Histological Tissue Features by Multi-dimensional Microscopic Molecular Profiling</td>
<td>BO – 3</td>
<td>51</td>
</tr>
<tr>
<td>Last Name</td>
<td>First Name</td>
<td>Abstract Title</td>
<td>Session</td>
<td>Page #</td>
</tr>
<tr>
<td>-------------------</td>
<td>------------</td>
<td>--------------------------------------------------------------------------------</td>
<td>---------</td>
<td>--------</td>
</tr>
<tr>
<td>Robinton</td>
<td>Daisy</td>
<td>Generating Genetically Modified Mice Using CRISPR/Cas9-Mediated Genome Engineering Technology</td>
<td>T</td>
<td>61</td>
</tr>
<tr>
<td>Robitaille</td>
<td>Aaron</td>
<td>Quantitative Label-Free Proteomics of Small Populations of Hemogenic Endothelium and Hematopoietic Progenitor Cells Derived From ESCs and Embryos</td>
<td>T</td>
<td>64</td>
</tr>
<tr>
<td>Rodríguez-Martínez</td>
<td>José</td>
<td>DNA Binding Specificity Determinants of the Human Pluripotency Transcription Factors Nanog and SOX2</td>
<td>BO – 3</td>
<td>25</td>
</tr>
<tr>
<td>Seita</td>
<td>Jun</td>
<td>Global-Scale Meta-Analysis Based Gene Expression Profiling Platform: Gene Expression Commons</td>
<td>T</td>
<td>56</td>
</tr>
<tr>
<td>Sharma</td>
<td>Arun</td>
<td>Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes as a Model for Coxsackievirus B3-Induced Viral Myocarditis</td>
<td>BO – 4</td>
<td>41</td>
</tr>
<tr>
<td>Sheehy</td>
<td>Sean</td>
<td>Heart-on-a-Chip: An <em>in vitro</em> Model for the Study of Myocardial Function</td>
<td>T</td>
<td>106</td>
</tr>
<tr>
<td>Silberstein</td>
<td>Lev</td>
<td>Proximity-Based Single Cell Analysis of the Bone Marrow Niche Identifies Interleukin-18 as a Quiescence Regulator of Early Hematopoietic Progenitors</td>
<td>BO – 1</td>
<td>80</td>
</tr>
<tr>
<td>Sim</td>
<td>Xiuli</td>
<td>A Transcriptome Profiling Approach to Define the Stages of Megakaryocyte Maturation</td>
<td>BO – 1</td>
<td>19</td>
</tr>
<tr>
<td>Slukvin</td>
<td>Igor</td>
<td>Successful Application of Induced Pluripotent Stem Cell Technology for <em>de novo</em> Generation Of Leukemia Stem-Like Cells and Discovery of Novel Leukemia Stem Cell Survival Factor</td>
<td>BO – 3</td>
<td>26</td>
</tr>
<tr>
<td>Termglinchan</td>
<td>Vittavat</td>
<td>Genome Editing of Isogenic Human Induced Pluripotent Stem Cells Recapitulates Long QT Phenotype for Drug Testing</td>
<td>BO – 1</td>
<td>42</td>
</tr>
<tr>
<td>Tian</td>
<td>Ying</td>
<td>A MicroRNA Pathway That Promotes Cardiomyocyte Proliferation and Cardiac Regeneration by Inhibiting Hippo Signaling</td>
<td>BO – 2</td>
<td>85</td>
</tr>
<tr>
<td>Turcotte</td>
<td>Raphaël</td>
<td>Defining Stem and Stromal Cell Couples in Hematopoiesis</td>
<td>BO – 4</td>
<td>81</td>
</tr>
<tr>
<td>Turcotte</td>
<td>Raphaël</td>
<td>Optical Platform for Intravital Characterization of Bone Marrow Stem and Progenitor Cells</td>
<td>T</td>
<td>82</td>
</tr>
<tr>
<td>Last Name</td>
<td>First Name</td>
<td>Abstract Title</td>
<td>Session</td>
<td>Page #</td>
</tr>
<tr>
<td>-----------</td>
<td>------------</td>
<td>---------------------------------------------------------------------------------</td>
<td>---------</td>
<td>--------</td>
</tr>
<tr>
<td>Vo</td>
<td>Karen</td>
<td>Using an iPSC Model of Jacobsen Syndrome to Study the Importance of FLI1 and ETS1 in Megakaryopoiesis</td>
<td>BO – 4</td>
<td>20</td>
</tr>
<tr>
<td>Wu</td>
<td>Haodi</td>
<td>Epigenetic Activation of Phosphodiesterase Subtypes Lead to Compromised Beta-Adrenergic Signaling in Induced Pluripotent Stem Cell-Derived Cardiomyocytes from Dilated Cardiomyopathy Patients</td>
<td>BO – 3</td>
<td>43</td>
</tr>
<tr>
<td>Yakubov</td>
<td>Eduard</td>
<td>Optimized Plasmid-Based Approach for Generation of High Quality Modified Messenger RNA</td>
<td>T</td>
<td>69</td>
</tr>
<tr>
<td>Yang</td>
<td>Wenli</td>
<td>Generation of a Lung Stem Cell “Tool Kit” for Derivation of Functional Lung Epithelial Lineages</td>
<td>BO – 2</td>
<td>86</td>
</tr>
<tr>
<td>Yzaguirre</td>
<td>Amanda</td>
<td>3-Dimensional Imaging: A Tool to Study Stem Cell Development in vivo</td>
<td>T</td>
<td>87</td>
</tr>
<tr>
<td>Zimmerlin</td>
<td>Ludovic</td>
<td>Optimized Rewiring of WNT and ERK Pathways is Sufficient for Stable Conversion of Human Pluripotent Stem Cells to a Naïve Ground State of Pluripotency</td>
<td>P</td>
<td>31</td>
</tr>
</tbody>
</table>