2012 Weinstein Cardiovascular Development Conference

May 2nd - 4th, 2012
Sheraton Chicago Hotel and Towers
Chicago, IL USA

Weinstein Cardiovascular Development Conference 2012

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Welcome

Welcome to the 2012 Chicago Weinstein meeting!

The organizing committee would like to welcome you to Chicago. This is the 19th Weinstein meeting and the first time it is being held in Chicago. Chicago is a world-class city with a lot to offer and we hope that everyone will have time to explore our city. On the river, very close to the Sheraton Hotel, is where the Architectural Boat Tours embark. This is an excellent way to see the tall buildings in Chicago. While most of you will be able to see downtown Chicago, the various outlying neighborhoods are also exceptional and we hope you can find time to visit them. Pilsen, Devon, Wicker Park, Logan Square, Bucktown, South Loop, Old Town, Oak Park and many others are awaiting you!

This year's meeting is hosted by members of two institutions: The University of Chicago and Northwestern University. Each institution has made significant contribution to the field of cardiac development. Since our institutions are located across the city of Chicago, the meeting will be held at the centrally located Sheraton Chicago Hotel & Towers located on the Chicago River, just east of the Magnificent Mile. This is an outstanding hotel that we hope you will enjoy during your stay.

We have tried to organize the meeting with great care to what we feel are the most timely and relevant areas of cardiac development. In addition, following the tradition of the Weinstein meeting, we organized most of the platform presentations so that trainees would be at the forefront of the meeting. New this year, we have added moderated poster discussions so that attendees can see selected posters and engage in lively, informal conversations about the topics covered. We want this meeting to be as interactive as possible to promote the free exchange of ideas.

The meeting Banquet will be on Thursday at the House of Blues, instead of the traditional Saturday night affair with the hope that more of you will be able to attend. The meeting will conclude on Friday night with our keynote speaker, Cliff Tabin. Persons then staying on will have the weekend to explore Chicago.

We hope that you enjoy the 19th Weinstein conference,

Sincerely,

The Weinstein Organizing Committee,

Eric Svensson, M.D., Ph.D., University of Chicago, Chairman

Robert Dettman, Ph.D., Northwestern University

Akira Imamoto, Ph.D., University of Chicago

Tsutomu Kume, Ph.D., Northwestern University

Elizabeth McNally, M.D., Ph.D., University of Chicago

Ivan Moskowitz, M.D., Ph.D., University of Chicago

Marcelo Nobrega, M.D., Ph.D., University of Chicago

Hans-Georg Simon, Ph.D., Northwestern University

General Announcements

Oral Presentations

Presentations will be held in the Sheraton Ballroom IV and V on the 4th level of the hotel. Each talk is scheduled for 15 minutes. This includes 12 minutes for the presentation and 3 minutes for questions. Please stay on time!

Moderated Poster Discussions

Two sessions will be held concurrently in River Exhibition Hall A during each general poster session of the meeting as detailed in the meeting schedule. These sessions will be directed by a moderator, and audience participation is encouraged!

Posters

All posters will be on display on Wednesday, Thursday, and Friday in River Exhibition Hall A on the first level of the hotel. Poster set-up begins on Wednesday at 5:40pm and must be removed by 4pm on Friday. Posters are numbered according to the abstract book. Authors of even numbered abstracts should be present at their poster on Wednesday evening's poster session, and authors of odd numbered abstracts should be present at the Thursday afternoon session.

Business Meeting

The Business meeting will be held in the Ohio room on the second level of the hotel at 12:30 pm on Thursday. At this time we will select a site for the 2015 meeting. All are welcome to participate.

Evaluations

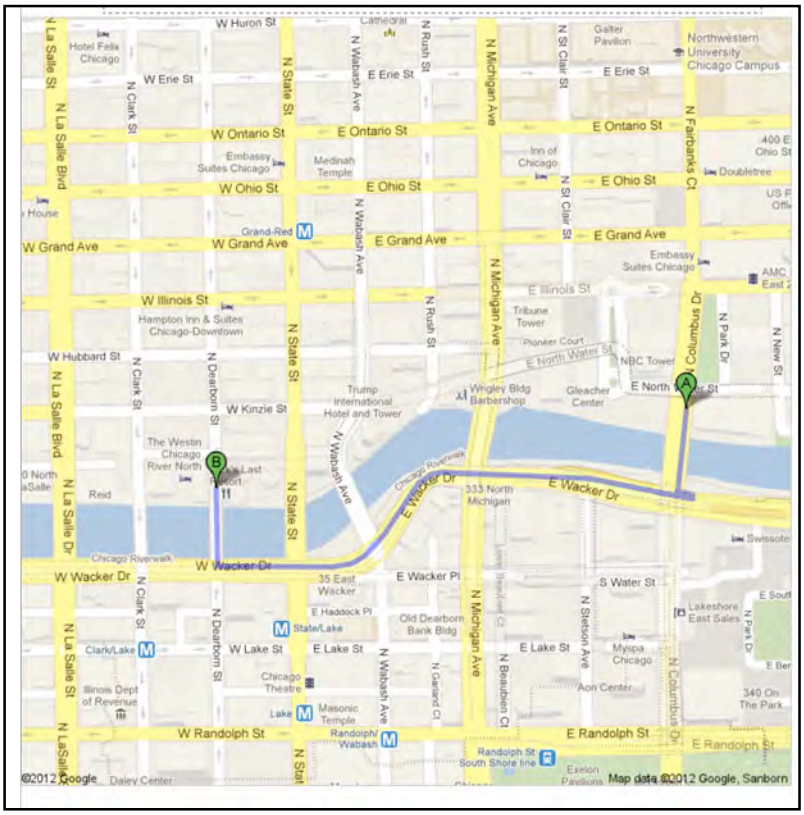
Please fill out and return the evaluation forms available at the registration desk. These forms provide important feedback to the organizers and help the future organizers improve the meeting and address any areas of concern.

Drink Tickets and Catering

In your registration materials, you will find two sets of drink tickets. The **white** tickets are for purchasing drinks during the poster session on Wednesday evening. The **red** tickets are for purchasing drinks during the banquet at the House of Blues. Lost tickets will not be replaced.

Banquet at the House of Blues

This year's meeting banquet will start at 6pm on Thursday evening at the House of Blues, 329 North Dearborn Street in downtown Chicago. The House of Blues is an easy 15-minute walk from the hotel as detailed on the map ("A" marks the Sheraton Hotel, "B" marks the House of Blues).

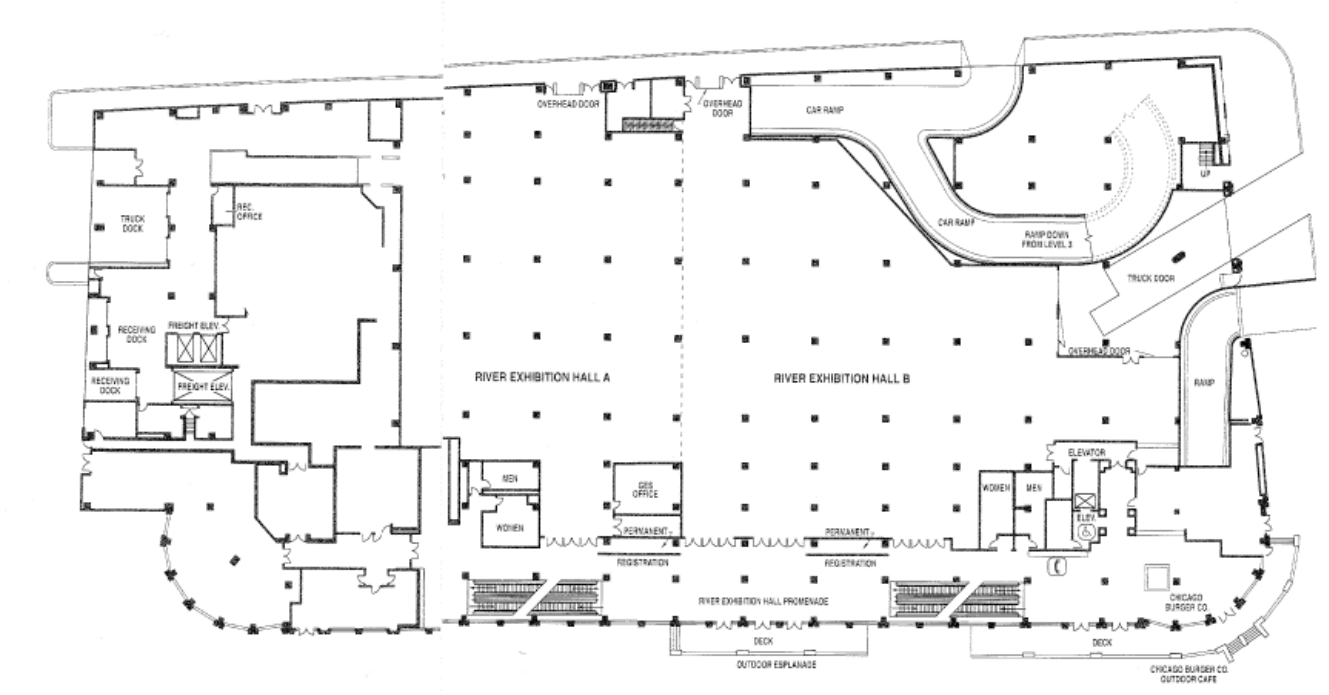


Acknowledgements

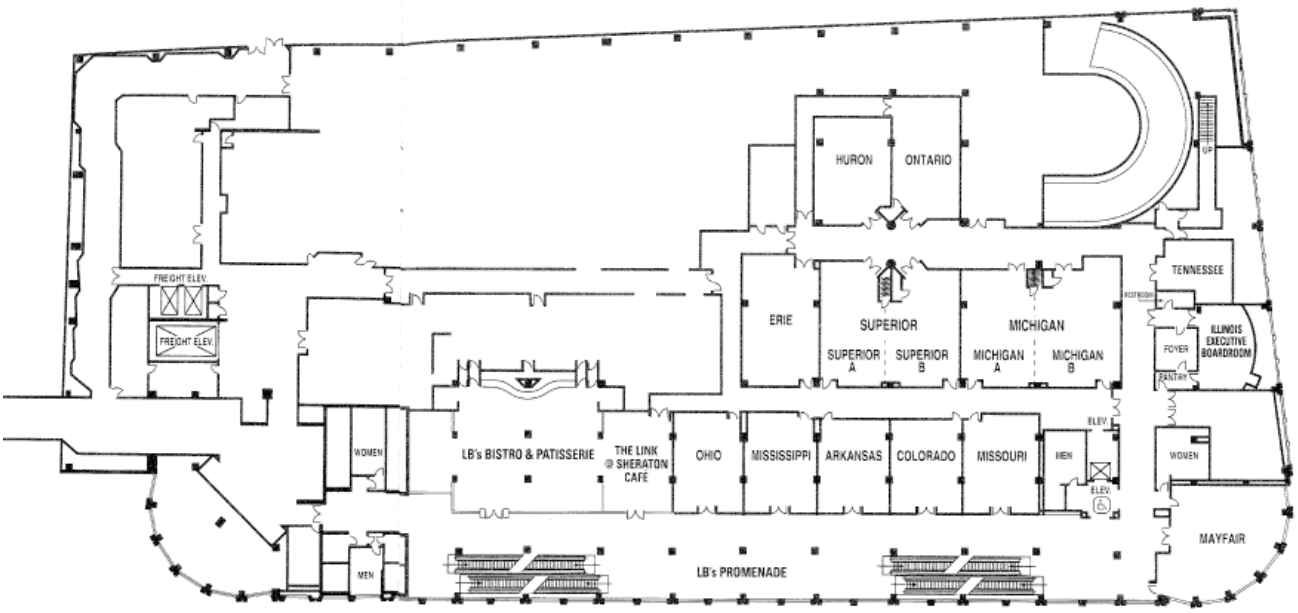
We are grateful to Ms. Tharrie Daniels for her administrative support of this meeting.

Sheraton Hotel Floor Plans

Level One – Poster Presentations: River Hall A

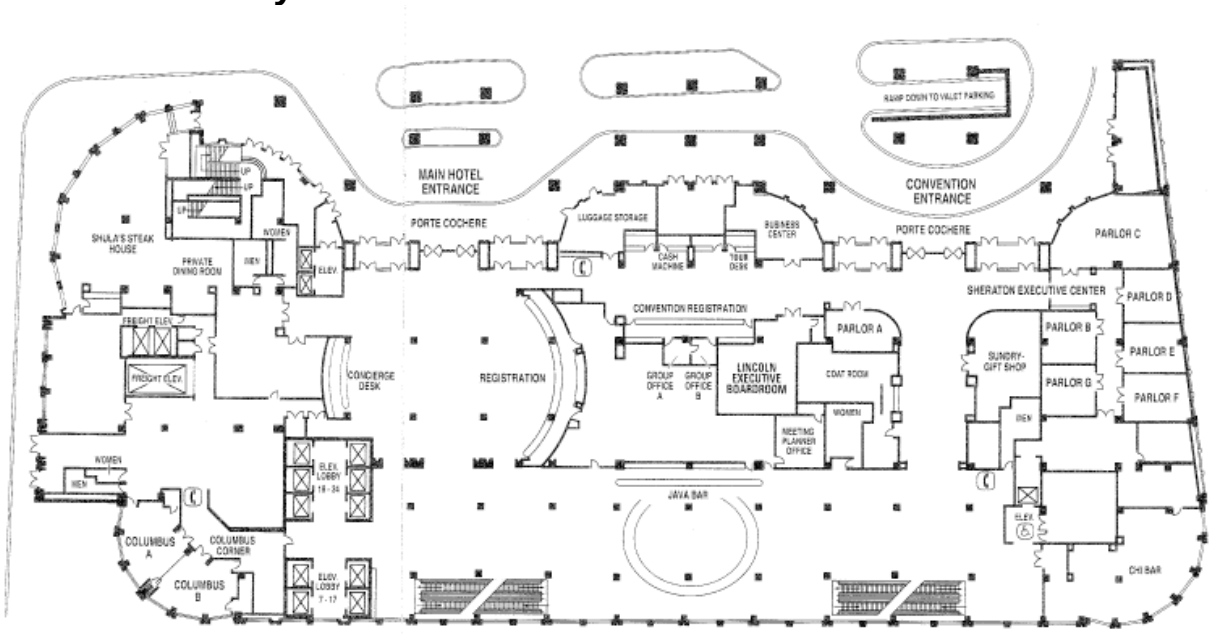


Level Two – Business Meeting: Ohio Room

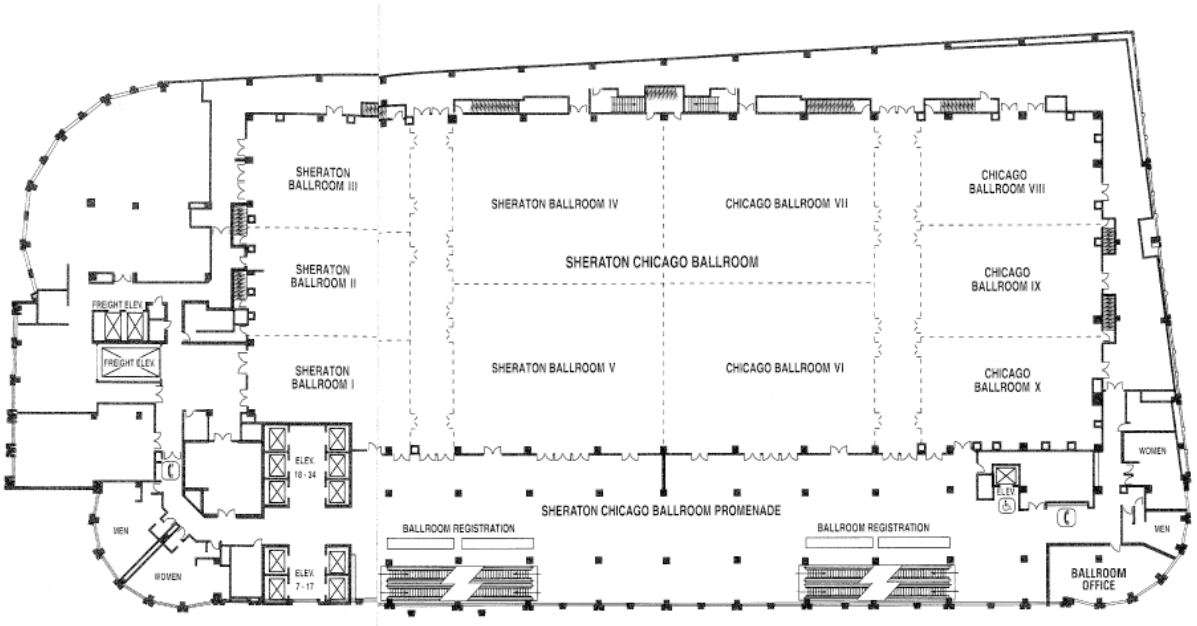


Sheraton Hotel Floor Plans

Level Three - Lobby



Level Four – Oral Presentations: Ballroom IV and V



Condensed Conference Schedule

Wednesday, May 2nd

12:00 pm	**Meeting Check-in**
2:00 pm	**Opening Remarks**
2:10-3:40 pm	**Platform Session 1: Human Genetics/CHD Models**
3:40-4:10 pm	**Coffee Break**
4:10-5:40 pm	**Platform Session 2: Cardiomyocyte Growth and Regeneration**
5:40-9:00 pm	**Poster Session 1 and Reception**
6:00-7:00 pm	**Moderated Poster Discussion 1: Cardiac Regeneration**
6:00-7:00 pm	**Moderated Poster Discussion 2: Human Genetics & Disease Models**

Thursday, May 3rd

7:00-8:30 am	**Continental Breakfast**
8:30-10:00 am	**Platform Session 3: Genomics and Gene Regulation**
10:00-10:30 am	**Coffee Break**
10:30-12:00 am	**Platform Session 4 Neural Crest and Vascular Development**
12:00-1:30 pm	**Lunch**
12:30-1:30 pm	**Weinstein Business Meeting**
1:30-4:00 pm	**Poster Session 2**
2:30-3:30 pm	**Moderated Poster Discussion 3: Transcriptional Regulation**
2:30-3:30 pm	**Moderated Poster Discussion 4: Coronary Artery Development**
4:00-5:30pm	**Platform Session 5: Coronaries, Epicardium, and Conduction System**
6:00-10:00 pm	**Banquet at the House of Blues**

Friday, May 4th

7:00-8:30 am	**Continental Breakfast**
8:30-10:00 am	**Platform Session 6: Early Heart Formation and Progenitors**
10:00-10:30 am	**Coffee Break**
10:30-12:00 pm	**Platform Session 7: Chamber Development**
12:00-1:30 pm	**Lunch**
1:00-3:30 pm	**Poster Session 3**
2:15-3:15 pm	**Moderated Poster Discussion 5: Valve Calcification & Development**
2:15-3:15 pm	**Moderated Poster Discussion 6: Stem Cells**
3:30-5:00 pm	**Platform Session 8: Valve Development**
5:00-6:00 pm	**Keynote Speaker – Dr. Cliff Tabin**
6:00-6:15 pm	**Presentation of Next Year's Venue** **Closing Remarks**

Conference Schedule

Wednesday, May 2nd

12:00 pm **Meeting Check-in**

2:00 pm **Opening Remarks**

2:10-3:40 pm Platform Session 1: Human Genetics/CHD Models

Session Chairs: Beth McNally, University of Chicago
Bernice Morrow, Albert Einstein College of Medicine

2:10 pm **S1.1** **Deletion of the extracellular matrix protein *Adamtsl2* results in a inter-ventricular septal defect in mice**
Dirk Hubmacher, Lauren W.Wang, Suneel S. Apte
Lerner Research Institute, Cleveland Clinic

2:25 pm **S1.2** **Complex trait analysis of ventricular septal defects caused by *Nkx2-5* mutation**
Claire E. Schulkey, Suk D. Regmi, Patrick Y. Jay
Washington University School of Medicine

2:40 pm **S1.3** **Functional analysis of novel ZIC3 mutations identified in patients with heterotaxy**
Jason Cowan, Muhammad Tariq, Stephanie M. Ware
University of Cincinnati

2:55 pm **S1.4** **The Genetic Basis of Isolated Tetralogy of Fallot**
Marcel Grunert, Cornelia Dorn, Markus Schueler, Ilona Dunkel, Jenny Schlesinger, Siegrun Mebus, Katherina Bellmann, Vladimir Alexi-Meskishvili, Sabine Klaassen, Katharina Wassilew, Bernd Timmermann, Roland Hetzer, Felix Berger, Silke R. Sperling
Max Planck Institute for Molecular Genetics

3:10 pm **S1.5** **An Excess of Deleterious Variants in VEGF-A Pathway Genes in Down Syndrome-Associated Atrioventricular Septal Defects**
Christine Ackerman, Adam E. Locke, Eleanor Feingold, Benjamin Reshey, Karina Espana, Janita Thusberg, Sean Mooney, Lora J. H. Bean, Kenneth J. Dooley, Clifford Cua, Roger H. Reeves, Stephanie L. Sherman, Cheryl L. Maslen
Oregon Health & Science University

3:25 pm **S1.6** **Identification of Novel Mutations in a Large Family with Different Forms of Congenital Heart Disease**
AC Fahed, RC Tanos, IG El-Rassy, FF Bitar, JG Seidman, CE Seidman, GM Nemer
Harvard Medical School and American University of Beirut, Beirut, Lebanon

3:40-4:10 pm **Coffee Break**

4:10-5:40 pm Platform Session 2: Cardiomyocyte Growth and Regeneration

Session Chairs: Hans-Georg Simon, Northwestern University
Da-Zhi Wang, Children's Hospital Boston

4:10 pm **S2.1** **Cardiac hypertrophy in LEOPARD Syndrome is caused by *PTPN11* loss-of-function activity in the developing endocardium**
Jessica Lauriol, Kimberly Keith, Boding Zhang, Roderick Bronson, Kyu-Ho Lee, and Maria I. Kontaridis
Beth Israel Deaconess Medical Center

4:25 pm **S2.2** **Epigenetic regulation of cardiac hypertrophy**
Qing-Jun Zhang, David Maloney, Anton Simeonov, and Zhi-Ping Liu
University of Texas Southwestern

4:40 pm **S2.3** **Regulation of striated muscle gene expression: A role for *Prox1* during cardiac and skeletal muscle development**
Louisa K. Petchey, Paul R. Riley
UCL Institute of Child Health, London

4:55 pm **S2.4** **CIP, a novel cardiac nuclear protein regulates cardiac function and remodeling**
Zhan-Peng Huang, Hee Young Seok, Jinghai Chen, and Da-Zhi Wang
Children's Hospital Boston

5:10 pm **S2.5** **Yap1 regulates cardiomyocyte proliferation through PIK3CB**
Zhiqiang Lin, Alexander von Gisea, Pingzhu Zhou, Jessie Buck, and William Pu
Children's Hospital Boston

5:25 pm **S2.6** **A Transitional Extracellular Matrix Instructs Epicardial-mediated Vertebrate Heart Regeneration**
Sarah Mercer, Claudia Guzman, Chia-ho Cheng, Shannon Odelberg, Ken Marx and Hans-Georg Simon
Northwestern University Feinberg School of Medicine

5:40-9:00 pm Poster Session 1 and Reception

6:00-7:00 pm **Moderated Poster Discussion 1: Cardiac Regeneration**
Moderated by Paul Riley

2.7 **Cardiomyocyte proliferation contributes to post-natal heart growth in humans**
Kevin Bersell, Mariya Mollova, Stuart Walsh, Jainy Savla, Tanmoy DasLala, Shin-Young Park, Leslie Silberstein, Cris dosRemedios, Dionne Graham, Steven Colan and Bernhard Kühn
Children's Hospital Boston

2.8 **Microarray and Ultrastructure Analyses of a Regenerative Myocardium**
Pooja Pardhanani, Jeremy Barth, Heather J. Evans-Anderson
Winthrop University

2.9 **Deciphering novel molecular mechanisms that facilitate cardiomyocyte de-differentiation and proliferation**
Rachel Sarig, Dana Rajchman, Yfat Yahalom, Elad Bassat, Benjamin Geiger, Eldad Tzahor
Weizmann Institute of Science, Israel.

2.10 **Tissue specific translational profiling during zebrafish heart regeneration**
Yi Fang, Vikas Gupta, Ravi Karra, Taylor Wahlig, Jennifer Holdway, Jinhu Wang, and Kenneth D. Poss
Duke University Medical Center

6:00-7:00 pm **Moderated Poster Discussion 2: Human Genetics & Disease Models**
Moderated by Cheryl Maslen

1.7 **Novel role of altered cardiac function in the progression of heart defects in Fetal Alcohol Syndrome (FAS)**
Ganga Karunamuni, Shi Gu, Lindsy Peterson, Zhao Liu, Yong Qiu Doughman, Kersti Linask, Michael Jenkins, Andrew Rollins, Michiko Watanabe
Case Western Reserve University

1.8 **Noonan syndrome associated RAF1 mutant evokes hypertrophic cardiomyopathy features in human cardiomyocytes in vitro.**
Malte Tiburcy, Nicole Dubois, Aleksander Hinek, Seema Mital, Darrell Kotton, Wolfram Zimmermann, Gordon Keller, Benjamin Neel, Toshiyuki Araki
University Health Network and Hospital for Sick Children, Toronto, Canada

1.9 **Engineering new mouse models to map dosage-sensitive genes in Down syndrome congenital heart defects**
Eva Lana-Elola, Sheona Watson-Scales, Amy Slender, Louisa Dunlevy, Mike Bennett, Timothy Mohun, Elizabeth M. C. Fisher, Victor L. J. Tybulewicz
National Institute for Medical Research, London

1.10 **Recovery of ENU induced mutations causing congenital heart disease using next-gen sequencing**
You Li, Sarosh Fatakia, Ashok Srinivasan, Histao Yagi, Rama Damerla, Bishwanath Chatterjee, Cecilia W. Lo
University of Pittsburgh School of Medicine

Thursday, May 3rd

7:00-8:30 am **Continental Breakfast**

8:30-10:00 am **Platform Session 3: Genomics and Regulation**
Session Chairs: Marcelo Nobrega, University of Chicago
Silke Sperling, Max Planck Institute

8:30 am **S3.1** **Isolation of nuclei from specific cardiac lineages in zebrafish for genome-wide and epigenomic analyses**
Bushra Gorsi and H. Joseph Yost
University of Utah

8:45 am **S3.2** **TU-tagging in mice defines dynamic gene expression programs in specific cardiovascular or other cell types within intact tissues**
Leslie Gay, Michael R. Miller, Vidusha Devasthali, P. Brit Ventura, Zer Vue, Heather L. Thompson, Sally Temple, Hui Zong, Michael D. Cleary, Kryn Stankunas, Chris Q. Doe
University of Oregon

9:00 am **S3.3** **Escape of Gene Body DNA Methylation in Cardiac Genes is Cooperative with the Cell Type-specific Expression Patterns**
Mayumi Oda, Shinji Makino, Hirokazu Enomoto, Ruri Kaneda, Shinsuke Yuasa and Keiichi Fukuda
Keio University, Tokyo, Japan

9:15 am **S3.4** **Epigenetic regionalization of the developing mouse heart**
Scott Smemo, Noboru J. Sakabe, Ivy Aneas, Marcelo A. Nobrega
University of Chicago

9:30 am **S3.5** **The novel mechanism of histone-chromatin regulation for cardiomyocytes regeneration**
Ryo Nakamaura, Kazuko Koshiba-Takeuchi, Yuko Tsukahara, Tetsuo Sasano, Mizuyo Kojima, Tetsushi Furukawa, Hesham A Sadek, Jun K Takeuchi
University of Tokyo, Tokyo, Japan

9:45 am **S3.6** **Drosophila microRNA-1 Genetically Interacts with little imaginal discs, a histone demethylase.**
Isabelle N. King
Gladstone Institute of Cardiovascular Disease, University of California, San Francisco

10:00-10:30 am **Coffee Break**

10:30-12:00 am Platform Session 4 Neural Crest and Vascular Development

Session Chairs: Tsutomu Kume, Northwestern University
Frank Conlon, University of North Carolina

10:30 am **S4.1** **Early neural crest-restricted Noggin over-expression results in congenital craniofacial and cardiovascular defects**
Zachary Neeb, Paige Snider and Simon J. Conway
Indiana University School of Medicine

10:45 am **S4.2** **The Role of Fibronectin - Integrin Signaling in Pharyngeal Microenvironment in Modulation of Cardiac Neural Crest Cell Development**
Dongying Chen and Sophie Astrof
Thomas Jefferson University

11:00 am **S4.3** **Slit3-Robo1/2 signalling controls cardiac innervation and ventricular septum development by regulating neural crest cell survival**
Mathilda T.M. Mommersteeg, William D. Andrews, Alain Chédotal, Vincent M. Christoffels, John G. Parnavelas
University College London, London

11:15 am **S4.4** **The neural crest contributes to coronary artery smooth muscle formation through endothelin signaling**
Yuichiro Arima, Sachiko Miyagawa-Tomita, Kazuhiro Maeda, Koichi Nishiyama, Rieko Asai, Ki-Sung Kim, Yasunobu Uchijima, Hisao Ogawa, Yukiko Kurihara and Hiroki Kurihara
University of Tokyo, Tokyo, Japan.

11:30 am **S4.5** **CASTOR directly regulates a novel Egfl7/RhoA pathway to promote blood vessel development and morphogenesis**
Marta Szmacinski, Kathleen Christine, Nirav Amin, Joan Taylor, Frank L. Conlon
University of North Carolina at Chapel Hill

11:45 am **S4.6** **Nephronectin regulates axial vein morphogenesis in zebrafish**
Chinmoy Patra, Filomena Ricciardi, Iva Nikolic, Mirko HH Schmidt, Benno Jungblut, Felix B. Engel
Max Planck Institute, Germany

12:00-1:30 pm **Lunch**

12:30-1:30 pm **Weinstein Business Meeting**

1:30-4:00 pm Poster Session 2

2:30-3:30 pm **Moderated Poster Discussion 3: Transcriptional Regulation**
Moderated by Sylvia Evans

3.7 **Mutual Regulation of *Nkx2.5* and *Fibulin-1* in the SHF Regulatory Network**
Anthony J. Horton, Marion A. Cooley, Waleed O. Twall, Victor M. Fresco, Boding Zhang, Christopher D. Clark, W. Scott Argraves and Kyu-Ho Lee
Medical University of South Carolina

3.8 **FOG-2 Mediated Recruitment of the NuRD Complex Regulates Cardiomyocyte Proliferation during Heart Development**
Audrey Jerde, Zhiguang Gao, Spencer Martens, Judy U. Early, Eric C. Svensson
The University of Chicago

3.9 **A strict lineage boundary between the first and second heart fields is defined by the contribution of the Tbx5 lineage**
Joshua D. Wythe, W. Patrick Devine, Kazuko Koshiba-Takeuchi, Kyonori Togi, Benoit G. Bruneau
Gladstone Institute of Cardiovascular Disease

3.10 **Tbx5-Hedgehog pathway is required in second heart field cardiac progenitors for atrial septation**
Linglin Xie, Andrew D. Hoffmann, Ozanna Burnicka-Turek, Joshua M. Friedland-Little, Ke Zhang, Ivan P. Moskowitz
The University of Chicago

2:30-3:30 pm **Moderated Poster Discussion 4: Coronary Artery Development**
Moderated by David Bader

5.7 **The BMP regulator BMPER is necessary for normal coronary artery formation**
Laura Dyer and Cam Patterson
University of North Carolina at Chapel Hill

5.8 **Epicardial chemokine signaling influences ventricular wall proliferation and coronary vasculogenesis**
Susana Cavallero, Hua Shen, Peng Li, Henry M. Sucov.
Keck School of Medicine, University of Southern California

5.9 **The role of Pod1/Tcf21 in epicardium-derived cells during cardiac development and fibrosis**
Caitlin M. Braitsch, Michelle D. Combs, Susan E. Quaggin, and Katherine E. Yutzey
Cincinnati Children's Hospital Medical Center

5.10 **Epicardial GATA factors regulate coronary endothelial migration via Sonic hedgehog signaling**
Kurt D. Kolander, Mary L. Holtz, and Ravi P. Misra
Medical College of Wisconsin

4:00-5:30pm Platform Session 5: Coronaries, Epicardium, and Conduction System

Session Chairs: Ivan Moskowitz, University of Chicago
William Pu, Children's Hospital Boston

4:00 pm **S5.1** **Ets-1 Regulates the Migration of Coronary Vascular Precursors and Coronary Endothelial Cell Proliferation**
Gene H. Kim, Saoirse S. McSharry, Zhiguang Gao, Ankit Bhatia, Judy Earley, and Eric C. Svensson
The Universtiy of Chicago

4:15 pm **S5.2** **Wnt signaling in the developing murine epicardium and epicardium-derived-cells (EPDCs)**
Carsten Rudat, Julia Norden, Makoto M. Taketo, Andreas Kispert
Hannover Medical School, Germany

4:30 pm **S5.3** **Determination of Cardiac Pacemaker Cell Origins Reveals a Critical Role for Wnt Signaling During their Cell Fate Specification.**
Michael Bressan, Gary Liu, and Takashi Mikawa
University of California San Francisco

4:45 pm **S5.4** **A Tbx5-Scn5a Molecular Network Modulates Function of the Cardiac Conduction System**
David E Arnolds, Fang Liu, Scott Smemo, John P Fahrenbach, Gene H Kim, Ozanna Burnicka-Turek, Elizabeth M McNally, Marcelo M Nobrega, Vickas V Patel, and Ivan P Moskowitz
The University of Chicago

5:00 pm **S5.5** **Mouse cardiac T-box targets reveal an Scn5a/10a enhancer functionally affected by genetic variation in humans**
Phil Barnett, Malou van den Boogaard, L. Y. Elaine Wong, Federico Tessadori, Martijn L. Bakker, Connie R. Bezzina, Peter A.C. 't Hoen, Jeroen Bakkers, Vincent M. Christoffels
University of Amsterdam

5:15 pm **S5.6** **Activation of voltage-dependent anion channel 2 suppresses Ca^{2+}-induced cardiac arrhythmia**
Hirohito Shimizu, Johann Schredelseker, Jie Huang, Kui Lu, Sarah Franklin, Kevin Wang, Thomas Vondriska, Joshua Goldhaber, Ohyun Kwon, Jau-Nian Chen
University of California, Los Angeles

6:00-10:00 pm **Banquet at the House of Blues**

Friday, May 4th

7:00-8:30 am **Continental Breakfast**

8:30-10:00 am **Platform Session 6: Early Heart Formation and Progenitors**
Session Chairs: Eric Svensson, University of Chicago
Robert Kelly, Aix-Marseille University

8:30 am **S6.1** **Quantitative analysis of polarity in 3D reveals local cell coordination in the embryonic mouse heart**
Jean-François Le Garrec, Chiara Ragni, Sorin Pop, Alexandre Dufour, Jean-Christophe Olivo-Marin, Margaret Buckingham and Sigolène Meilhac
Institut Pasteur, Paris, France

8:45 am **S6.2** **Clonally dominant cardiomyocytes direct heart morphogenesis**
Vikas Gupta and Kenneth D. Poss
Duke University Medical Center

9:00 am **S6.3** **$G\alpha_{13}$ is required for S1pr2-mediated myocardial migration by regulating endoderm morphogenesis**
Ding Ye, Songhai Chen, Fang Lin
University of Iowa

9:15 am **S6.4** **A Cdc42 associated genetic network directs heart lumen formation and morphogenesis in Drosophila**
Georg Vogler, Jiandong Liu and Rolf Bodmer
Sanford-Burnham Medical Research Institute, La Jolla, CA

9:30 am **S6.5** **Identification and characterization of a multipotent cardiac precursor**
W. Patrick Devine, Josh D. Wythe, Benoit G. Bruneau
Gladstone Institute of Cardiovascular Disease

9:45 am **S6.6** **Distinct origin and commitment of HCN4+ First Heart Field cells towards cardiomyogenic cell lineages**
Daniela Später, Monika Abramczuk, Jon Clarke, Kristina Buac, Makoto Sahara, Andreas Ludwig, Kenneth R. Chien
Massachusetts General Hospital

10:00-10:30 am **Coffee Break**

10:30-12:00 pm Platform Session 7: Chamber Development

Session Chairs: Akira Imamoto, University of Chicago
Deborah Yelon, University of California, San Diego

10:30 am **S7.1** ***Irx1a*** **Acts Downstream of** ***nkx*** **Genes in Maintaining Cardiac Chamber Identity in Zebrafish**
Sophie Colombo, Vanessa George, Thomas Schell, Lilianna Solnica-Krezel, Deborah Yelon, and Kimara L. Targoff
College of Physicians & Surgeons, Columbia University

10:45 am **S7.2** **Zebrafish second heart field development relies on early specification of progenitors and nkx2.5 function**
Burcu Guner-Ataman, Noelle Paffett-Lugassy, Meghan S. Adams, Kathleen R. Nevis, Caroline E. Burns, C. Geoffrey Burns
Massachusetts General Hospital

11:00 am **S7.3** **Notch1 mediated signaling cascades regulate cardiomyocyte polarity and ventricular wall formation.**
Wenjun Zhang, Hanying Chen, Momoko Yoshimoto, Jin Zhang, Mervin C Yoder, Nadia Carlesso, Weinian Shou
Indiana University School of Medicine

11:15 am **S7.4** **Mutations in the NOTCH pathway regulator MIB1 cause ventricular non-compaction cardiomyopathy**
Guillermo Luxán, Jesús C. Casanova, Beatriz Martínez-Poveda, Donal MacGrogan, Álvaro Gonzalez-Rajal, Belén Prados, Gaetano D'Amato, David Dobarro, Carlos Torroja, Fernando Martinez, José Luis Izquierdo-García, Leticia Fernández-Friera, María Sabater-Molina, Young-Y. Kong, Gonzalo Pizarro, Borja Ibañez, Constancio Medrano, Pablo García-Pavía, Juan R. Gimeno, Lorenzo Montserrat, Luis J. Jiménez-Borreguero & José Luis de la Pompa
Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain

11:30 am **S7.5** **The early role of** ***Tbx1*** **in anterior and posterior second heart field cells**
M. Sameer Rana, Karim Mesbah, Jorge N. Dominguez, Niels Hofschreuder, Virginia E. Papaioannou, Antoon F. Moorman, Vincent M. Christoffels, Robert G. Kelly
Aix-Marseille University, Marseille, France

11:45 am **S7.6** **Retinol Dehydrogenase 10: Roles in Embryo Pharyngeal Patterning and a Model to Understand How Retinoid Gradients Prevent Congenital Birth Defects**
Muriel Rhinn, Pascal Dollé, Richard Finnell, and Karen Niederreither
The University of Texas, Austin

12:00-1:30 pm **Lunch**

1:00-3:30 pm Poster Session 3

2:15-3:15 pm **Moderated Poster Discussion 5: Valve Calcification & Development**
Moderated by Andy Wessels

8.7 **Analysis of TGFβ3 function in valve remodeling and aortic valve calcification**
Alejandra C Encinas, Robert Hinton, and Mohamad Azhar
Indiana University School of Medicine

8.8 **Nuclear exclusion of Sox9 in valve interstitial cells is associated with calcific phenotypes in heart valves**
Danielle J. Huk, Harriet Hammond, Robert. B. Hinton, and Joy Lincoln
The Research Institute at Nationwide Children's Hospital, Columbus

8.9 **Runx2 isoforms have divergent roles in development and pathology in the heart**
Andre L.P. Tavares, Jesse A. Brown, Katarina Dvorak and Raymond B. Runyan
University of Arizona

8.10 **3D cardiac valve tissue reconstruction and quantitative evaluations of tissue & cell phenotypes in the *Pdlim7* knock-out mouse.**
Rudyard W. Sadleir, Robert W. Dettman, Jennifer Krcmery, Brandon Holtrup, and Hans-Georg Simon
Northwestern University Feinberg School of Medicine

2:15-3:15 pm **Moderated Poster Discussion 6: Stem Cells**
Moderated by Richard Harvey

6.7 **Dynamic Mesp1 regulation directs cardiac myocyte formation in embryonic stem cells**
Yu Liu, Xueping Xu, Austin Cooney, Robert Schwartz
University of Houston

6.8 **Human amniocytes contain subpopulations of stem cells that have a repressed cardiogenic status**
Colin T. Maguire, Bradley Demarest, Jennifer Akiona, Jonathon Hill, James Palmer, Arthur R. Brothman, H. Joseph Yost, Maureen L. Condic
University of Utah Molecular Medicine Program

6.9 **The Homeobox Transcription Factor, Irx4, Identifies a Mutipotent, Ventricular-specific Cardiac Progenitor**
Daryl Nelson, Joshua Trzebiatowski, Erin Willing, Pearl Hsu, Kevin Schoen, Kevin Liberko, Matthew Butzler, Pat Powers, Manorama John, Timothy Kamp, Gary Lyons,
University of Wisconsin-Madison

6.10 **Elastin regulates smooth muscle cell differentiation in human induced-pluripotent stem cells in Williams-Beuren syndrome**
Caroline Kinnear, Wing Y. Chang, Karen Kennedy, Aleksander Hinek, Tadeo Thompson, Shahryar Khattak, Sylvie Gervier, Maryam Niapour, Naila Mahmut, Yanting Wang, Gordon Keller, William L. Stanford, James Ellis, Seema Mital
Hospital for Sick Children, Toronto, Canada

3:30-5:00 pm — Platform Session 8: Valve Development

Session Chairs: Robert Dettman, Northwestern University
Joy Lincoln, Nationwide Children's Hospital

3:30 pm **S8.1** **Computational modeling of endocardial cell activation during AV valve formation**
A.K. Lagendijk, A. Szabo, R. Merks, and J. Bakkers
Hubrecht Institute and University Medical Center, Utrecht, The Netherlands

3:45 pm **S8.2** **Tie1 is Required for Semilunar Valve Form and Function**
Chris Brown, Xianghu Qu, Kate Violette, M-K Sewell, W. David Merryman, Bin Zhou, and H. Scott Baldwin
Vanderbilt University

4:00 pm **S8.3** **In Vivo Reduction Of Smad2 Concomitant With Proteoglycan Accumulation Results In High Penetrance Of Bicuspid Aortic Valves**
Loren Dupuis, Robert B. Hinton and Christine B. Kern
Medical University of South Carolina

4:15 pm **S8.4** **Filamin-A Regulates Tissue Remodeling of Developing Cardiac Valves via a Novel Serotonin Pathway**
Kimberly Sauls, Annemarieke de Vlaming, Katherine Williams, Andy Wessels, Robert A. Levine, Susan A Slaugenhaupt, Roger R. Markwald, Russell A. Norris
Medical University of South Carolina

4:30 pm **S8.5** **Epicardially-derived Fibroblasts Preferentially Contribute to the Parietal Leaflets of the Atrioventricular Valves in the Murine Heart**
Andy Wessels, Maurice J. B. van den Hoff, Richard F. Adamo, Aimee L. Phelps, Marie M. Lockhart, Kimberly Sauls, Laura E. Briggs, Russell A. Norris, Bram van Wijk, Jose M. Perez-Pomares, Robert W. Dettman, and John B. E. Burch
Medical University of South Carolina

4:45 pm **S8.6** **Endothelial nitric oxide signaling regulates Notch1 in aortic valve development and disease**
Kevin Bosse, Chetan P. Hans, Ning Zhao, Sara N. Koenig, Nianyuan Huang, Anuradha Guggilam, Ge Tao, Pamela A. Lucchesi, Joy Lincoln, Brenda Lilly and Vidu Garg
Nationwide Children's Hospital, Columbus

5:00-6:00 pm — Keynote Speaker – Dr. Cliff Tabin

Harvard Medical School

6:00-6:15 pm **Presentation of Next Year's Venue**
Closing Remarks

END OF MEETING

Keynote Presentation

Clifford J. Tabin, PhD
George Jacob and Jacqueline Hazel Leder Professor
Chairman of the Department of Genetics
Harvard Medical School
Boston, Massachusetts

Clifford J. Tabin, PhD, professor and chairman of the Department of Genetics at Harvard Medical School, is considered one of the world leaders in current developmental biology. His pioneering work on Sonic hedgehog signaling (1) and the molecular pathway that controls the left and right sides of the body (2,3), revealed basic mechanistic insights into the emergence of anatomical form and organization during vertebrate development. These findings have contributed critically to our understanding of congenital limb malformations and asymmetry-related defects, including those of the heart.

Following the footsteps of his father, Cliff Tabin attended the University of Chicago as an undergraduate and expected to launch a career in physics. However, by the time he began his PhD studies at the Massachusetts Institute of Technology (MIT) in Cambridge, the recombinant DNA molecular biology revolution was beginning. “It was clear that this new technology was going to allow us to do things that no one had ever done before and understand things in a totally new way,” Cliff Tabin recalled. He turned his focus, joined the laboratory of Dr. Robert Weinberg, a pioneer in the study of retroviruses and oncogenes to do his doctoral thesis. Cliff Tabin was the first researcher at MIT to construct a retrovirus vector, and during his studies he uncovered the genetic changes in oncogenic RAS, inserted mutant RAS into his retrovirus vector, and demonstrated that it could cause cancer in mice (4).

Over the past two decades the Tabin lab has contributed significantly to our understanding of the development of a wide variety of tissues in the embryo, including the forming skeleton. However, he did not stop there, he is also a leader in uncovering how changes in the regulation of genes during embryonic growth is altered through the course of animal evolution to produce the variety of forms seen in the natural world. For example, he sent students to collect data from finches in the Galapagos and in translating the findings to the chicken embryo, they identified that the expression of Bmp4 and calmodulin determines differences in beak size (5,6). Another expedition led students to central Mexico to collect blind fish from caves, as “Caves provide a unique environment for evolution,” he said. He even sent students as far as the Gobi Desert to collect organisms for his evolutionary studies. However, Cliff Tabin has never been to those places himself. “I enjoy the photographs and live vicariously,” he said.

“I have never been to Nepal, either,” Cliff Tabin added. Nevertheless, he followed through on an philanthropic opportunity his brother Geoff got him into, and became chairman of an international advisory board to the Patan University of Health Sciences, a new medical school that he and a group of Nepalese physicians established in Kathmandu, to train physicians to serve in rural districts. Cliff Tabin and his team developed the curriculum for the medical school, and he has been actively recruiting volunteers from Harvard to teach courses until the Nepalese faculty develops the expertise to take them over.

Cliff Tabin’s honors and awards number many and include the 2012 Conklin Medal of the Society for Developmental Biology and the 2008 March of Dimes Prize in Developmental Biology. Furthermore, he became an elected member of the National Academy of Sciences in 2007 and of the American Academy of Arts and Sciences in 2000, and he was the recipient of the National Academy of Sciences Award in Molecular Biology in 1999.

Weinstein Cardiovascular Development Conference Charter

Scope of the Conference

The Weinstein Cardiovascular Development Conference is an annual meeting for scientists investigating normal and abnormal development of the heart and vasculature as it may ultimately relate to human disease. It is a freestanding meeting, unaffiliated with any society or parent organization. Interested individuals or groups from host institutions organize it on a rotating basis. The intent of the meeting is to advance the overall field of cardiovascular development through the sharing of information and the facilitation of collaborative investigations. True to the vision of Dr. Constance Weinstein, who first organized this conference, the meeting is intended to include as many perspectives as possible. Investigators in any relevant area that can provide contributions to our understanding of heart and vascular development are welcome to contribute.

Organization of the Conference

In order to provide continuity and to maintain quality the conference, the participants of the 1998 meeting voted to form an organizing committee called the "Weinstein Committee". The makeup of the committee is composed of a single representative from each of the three previous local organizing committees, a single member from the current host site local organizing committee, and a single representative from each of the next two proposed meeting sites. In addition, two "At-Large" members, who are selected by a vote by the conference participants, will serve a three-year term. The charge to the Committee is to assist the local organizing committee with meeting arrangements and organization and to help secure funding. Any institution should have a maximum of one member serving on the Weinstein Committee at any given time.

In addition, the Committee is charged with soliciting and vetting nominations for future meeting sites and host institutions. Prospective host institutions should bid to host a future Weinstein meeting three years prior to the year that they desire to host the meeting. Prospective host institutions should submit a preliminary application to the two At-Large Weinstein Committee members at least one month prior to bidding to host a future meeting. The preliminary application should contain details of the prospective local organizing committee, prospective site for the meeting, and a fundraising plan. The purpose of early submission of a preliminary application is to allow the At-Large members of the committee to resolve any potential issues or missing details prior to review of the applications by the entire Weinstein Committee at the annual business meeting. The Weinstein Committee will evaluate all bids for feasibility to host the conference effectively in terms of fundraising and organizational and scientific capacity. The Weinstein Committee will select a maximum of three bids to be put to a vote the following day by all conference attendees. Meeting sites will be selected by vote such that the future local organizing committee will have a three-year lead-time. The Weinstein Committee may solicit additional applications from prospective host institutions as needed. In the event that multi-year funding is sought from the National Institutes of Health or other national sources, the Weinstein Committee will participate in this process.

Local Organizing Committee

To provide a varied flavor and the opportunity for new approaches, each host institution will form a local organizing committee to select a meeting venue and format and to participate in fundraising. The site should be selected for its potential to optimize informal communication and interaction. As a way to emphasize new and topical information, organizers from the host institution should select speakers from among the submitted abstracts. Scheduling should include opportunities for new voices and encourage the development of students, fellows, and younger faculty. Ample time for discussion is to be provided.

Obligations of the Participants

One of the most important aspects of the Weinstein Conference has been the willingness of the participants to share new and unpublished information. This has provided opportunities for the participants to devise new experiments and develop new hypotheses in a collaborative manner. It is expected that all participants will participate in a collegial and ethical manner with respect to information obtained at the Weinstein Conference. Permission should be obtained before disclosure of another investigator's unpublished data.

Similarly, investigators pursuing similar experiments should inform a presenter if the divulged information has a bearing on their own work. All participants in the conference should be willing to share their expertise and reagents in the collective advancement of the area of cardiovascular development.

Annual Business Meeting

Each Weinstein Conference will include time set aside for a business meeting and time for a subsequent vote on a future conference site by conference participants. At the Business Meeting, Weinstein Committee members may consider changes in the direction of the conference or its organization. At the 1999 meeting in Tucson, Arizona, this Charter was distributed to the participants and ratified. Its provisions commenced at the business meeting of the 1999 Tucson, Arizona Conference. The Charter will remain in effect until modified by a vote of the Weinstein Committee at the annual business meeting.

Weinstein Conference History

The Weinstein Cardiovascular Development Conference is a continuation of an annual meeting of investigators funded by three separate RFAs on Cardiac Development in 1986, 1988, and 1990 at the National Heart, Lung and Blood Institute of the National Institutes of Health. Grants funded under the RFA mechanism resulted in a total of 26 research programs in the area of Cardiac Development. A separate program of Specialized Centers in Research (SCORs) in Congenital Heart Disease resulted in the addition of investigators from the Universities of Iowa, Pennsylvania and Rochester. Investigators were brought together annually at the National Institutes of Health to discuss their latest research results and discuss potential areas of collaboration. The first meeting was held in a basement meeting room at the NIH and included approximately a dozen participants. These annual meetings at the NIH between 1986 and 1993 continued to grow as new research programs were funded and by word of mouth with collaborators. These meetings coincided with the emergence of molecular biological and genetic approaches to the investigation of heart development. Upon the expiration of the last of the RFA programs, there was a strong interest in keeping these collegial and informative meetings going. Independent meetings have been held every year since 1994.

Dr. Constance Weinstein of the NHLBI was instrumental in focusing the attention of the NIH on the need for research in this area. She obtained the funds for the RFA and SCOR programs and organized the intramural meetings held at the NIH. To honor Dr. Weinstein, in 1995, the meeting was formally named the Weinstein Cardiovascular Development Conference. Dr. Weinstein is retired from NHLBI but she attends these conferences to monitor progress in the field that she spent so much effort fostering.

Weinstein Conference Host Institutions and Sites

Medical University of South Carolina (Charleston, 1994)
University of Rochester (Rochester, 1995)
University of Pennsylvania (Philadelphia, 1996)
University of Cincinnati (Cincinnati, 1997)
Vanderbilt University (Nashville, 1998)
University of Arizona (Tucson, 1999)
Washington University (St. Louis, 2000)
University of Texas, Southwestern Medical School (Dallas, 2001)
University of Utah (Salt Lake City, 2002)
Harvard University (Cambridge, 2003)
Leiden University (Leiden, Netherlands, 2004)
University of Arizona (Tucson, 2005)
University of South Florida (Tampa/St. Petersburg, 2006)
Indiana University School of Medicine (Indianapolis, 2007)
Texas A&M University (Houston, 2008)
University of California, San Francisco (San Francisco, 2009)
University of Amsterdam, Netherlands (Amsterdam, 2010)
University of Cincinnati (Cincinnati, 2011)
University of Chicago and Northwestern University (Chicago, 2012)
University of Arizona (Tucson, 2013)
Centro Nacional de Investigaciones Cardiovasculares (Madrid, 2014)

2012 Weinstein Committee Members

(Term expiration indicated in parentheses)

National Weinstein Committee

Brian L. Black, Ph.D., University of California, San Francisco (*2012*)
Organizer, 2009 Weinstein Conference

Maurice van den Hoff, Ph.D, Heart Failure Research Center, Amsterdam (*2013*)
Organizer, 2010 Weinstein Conference

Katherine Yutzey, Ph.D., University of Cincinnati (*2014*)
Organizer, 2011 Weinstein Conference

Eric Svensson, M.D., Ph.D., University of Chicago (2015)
Organizer, 2012 Weinstein Conference

Brad Davidson, Ph.D., University of Arizona (2016)
Organizer, 2013 Weinstein Conference

Jose Luis De la Pompa, Ph.D., Centro Nacional de Investigaciones Madrid (2017)
Organizer, 2014 Weinstein Conference

Paul Riley, Ph.D., UCL- Institute of Child Health (2014)
Member at Large

Deborah Yelon, Ph.D., University of California, San Diego (2014)
Member at Large

Local Organizing Committee Members

Eric Svensson, M.D., Ph.D., University of Chicago, Chairman

Robert Dettman, Ph.D., Northwestern University

Akira Imamoto, Ph.D., University of Chicago

Tsutomu Kume, Ph.D., Northwestern University

Elizabeth McNally, M.D., Ph.D., University of Chicago

Ivan Moskowitz, M.D., Ph.D., University of Chicago

Marcelo Nobrega, M.D., Ph.D., University of Chicago

Hans-Georg Simon, Ph.D., Northwestern University

Tharrie Daniels, Administrative Assistant

Sponsors

The 2012 Weinstein Cardiovascular Development Conference is hosted by the University of Chicago and Northwestern University. Continuing Weinstein meeting support is also provided by a grant form the National Heart, Lung, and Blood Institute and the National Institute of Child Health and Human Development.

Additional financial support was provided by the following:

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Abstracts

Oral Sessions

Platform Session 1: Human Genetics/CHD Models

S1.1 Deletion of the extracellular matrix protein *Adamtsl2* results in a inter-ventricular septal defect in mice

Dirk Hubmacher, Lauren W.Wang, Suneel S. Apte
Department of Biomedical Engineering, Lerner Research Institute, Cleveland Clinic

Geleophysic dysplasia (GD) is a rare human genetic disorder presenting with short stature, joint contractures and thick skin. High morbidity, and frequently, juvenile mortality results from cardiac valvular and tracheo-pulmonary anomalies. Mutations in the extracellular proteins ADAMTSL2 or fibrillin-1 lead to recessive or dominant GD, respectively. Excess TGFβ signaling was described in cells derived from GD patients and may constitute a major pathogenetic mechanism. ADAMTSL2 directly interacts with latent transforming growth factor-β binding protein (LTBP)-1 and fibrillin-1. Fibrillin-1 and -2 assemble into microfibrils and modulate extracellular TGFβ and BMP signaling. The *Adamtsl2* gene was deleted in mice by an intragenic IRES-*lacZ-neomycin* cassette, which also provided an expression reporter. *Adamtsl2* deletion resulted in neonatal lethality due to an inter-ventricular septal defect (VSD) and lung abnormalities. β -gal reporter staining showed *Adamtsl2* expression within the embryonic heart and lung, consistent with the observed anomalies. Cardiac expression was primarily localized to the crest of the inter-ventricular septum, the atrio-ventricular valve annulus and coronary vessels. Histology revealed a VSD in 80% of the mice with enhanced, localized fibrillin-1 and -2 staining at the crests of the inter-ventricular septum. In vitro, recombinant ADAMTSL2 bound to both fibrillin-1 and -2, and accelerated the biogenesis of fibrillin-1 microfibril formation in fetal bovine nuchal ligament cells, while blocking fibrillin-2 assembly. This suggests that ADAMTSL2 may provide a mechanism to actively regulate the ratio of fibrillin-1 to fibrillin-2 in microfibrils, with potential consequences for TGFβ/BMP signaling. These studies suggest a new role for extracellular matrix in regulation of cardiac development.

S1.2 Complex trait analysis of ventricular septal defects caused by *Nkx2-5* mutation

Claire E. Schulkey, Suk D. Regmi, Patrick Y. Jay
Departments of Pediatrics and Genetics, Washington University School of Medicine, St. Louis, MO.

Congenital heart disease is a complex trait. The same deleterious mutation typically causes widely varying presentations because additional, poorly defined factors modify phenotype. Historically, the modifiers have received less scrutiny than the causes. Nevertheless, the factors, especially ones that reduce risk, may suggest preventive strategies that a focus on monogenic causes has not. Hence, we assessed the role of genetic and environmental factors on the incidence of ventricular septal defects (VSD) caused by an *Nkx2-5* heterozygous knockout mutation. We phenotyped >3100 hearts from a second generation intercross of the inbred mouse strains C57BL/6 and FVB/N. Genetic linkage analysis mapped loci with LOD scores of 5-7 on chromosomes 6, 8 and 10 that influence the susceptibility to membranous VSD in *Nkx2-5*$^{+/-}$ animals. The chromosome 6 locus overlaps one for muscular VSD susceptibility. Multiple logistic regression analysis for environmental variables revealed that maternal age is correlated with the risk of membranous and muscular VSD in *Nkx2-5*$^{+/-}$ but not wild-type animals. The maternal age effect is unrelated to aneuploidy or a genetic polymorphism in the progeny. Ovarian transplant experiments between young and old females indicate that the basis of the maternal age effect resides in the mother and not the oocyte. Experiments to characterize a potential metabolic basis of the effect strongly suggest that the VSDs caused by *Nkx2-5* mutation can be prevented. Enumerable factors contribute to the presentation of a congenital heart defect. Their characterization in a mouse model offers the opportunity to define unanticipated pathways and to develop strategies for prevention.

S1.3 Functional analysis of novel ZIC3 mutations identified in patients with heterotaxy

Jason Cowan, Muhammad Tariq, Stephanie M. Ware
University of Cincinnati

Loss of function mutations in the zinc finger in cerebellum 3 (ZIC3) transcription factor result in heterotaxy, a condition characterized by abnormal left-right positioning of thoraco-abdominal organs and a wide variety of congenital anomalies, particularly of the cardiovascular system. Mutations in *ZIC3* have been reported in approximately 75% of all familial and 1% of all sporadic heterotaxy cases and have been associated with VACTERL, a constellation of malformations phenotypically overlapping heterotaxy. The presence of a polyalanine (polyA) expansion in one patient with VACTERL is of particular interest as pathogenic expansions have been identified in several other developmentally critical transcription factors, including *ZIC2.* To further define the incidence and functional significance of *ZIC3* mutations in heterotaxy, coding regions and splice junctions were screened in 200 unrelated heterotaxy patients. Nine mutations (8 novel) were identified, including a single alanine expansion (c.insGCC159-160). Functional analyses were supplemented by 4 additional recently reported *ZIC3* mutations. Aberrant ZIC3 cytoplasmic localization was observed for mutations spanning multiple nuclear localization domains between amino acids 155 and 318 and correlated with decreased transactivation of a luciferase reporter. A missense mutation within zinc finger 5 (p.A447G) surprisingly increased luciferase transactivation, despite elevated levels of cytoplasmic ZIC3. Neither polyA tract expansion differed significantly from wildtype with respect to either luciferase transactivation or ZIC3 subcellular localization. These analyses collectively indicate a higher than expected percentage of *ZIC3* mutations in patients with sporadic heterotaxy and suggest alternative pathogenesis of some *ZIC3* mutations, notably those within the polyA tract.

S1.4 The Genetic Basis of Isolated Tetralogy of Fallot

Marcel Grunert[1,2,], Cornelia Dorn[1,2,3,*], Markus Schueler[1,2], Ilona Dunkel[1], Jenny Schlesinger[2], Siegrun Mebus[4], Katherina Bellmann[2,3], Vladimir Alexi-Meskishvili[5], Sabine Klaassen[6], Katharina Wassilew[7], Bernd Timmermann[8], Roland Hetzer[5], Felix Berger[4], Silke R. Sperling[1,2,3]*

[1]Group of Cardiovascular Genetics, Max Planck Institute for Molecular Genetics, [2]Department of Cardiovascular Genetics, Experimental and Clinical Research Center, Charité – University Medicine Berlin and Max Delbrück Center for Molecular Medicine, [3]Department of Biology, Chemistry and Pharmacy at Freie Universität of Berlin, [4]Department of Pediatric Cardiology, [5]Department of Cardiac Surgery and [7]Department of Pathology at German Heart Center Berlin, [6]National Registry of Congenital Heart Disease, [8]Next Generation Service Group at Max Planck Institute for Molecular Genetics, [*]shared authorship

Background

Tetralogy of Fallot (TOF) represents 10% of congenital heart disease, which are the most common birth defect in human. The majority of TOF are isolated non-syndromic cases of unknown precise cause, which applies for the majority of congenital heart disease.

Methods

We performed targeted resequencing of over 1,000 genes and microRNAs as well as whole transcriptome and miRNome analysis in TOF cases, parents and controls using next-generation sequencing techniques. We defined a set of TOF genes with deleterious variations and which are mutated at a higher rate in TOF subjects compared to healthy controls.

Results

We identified an oligogenic architecture underlying TOF, which discriminate TOF cases from controls. On average, TOF subjects show a combination of novel and inherited variations in five genes. The majority of genes has known association with human cardiac disease and/or shows a cardiac phenotype in mouse mutants. Seven genes are novel and have not yet been linked to a cardiac phenotype. Functionally interacting yet individual mutations lead to the same phenotypic outcome during development. The majority of TOF genes shows continuous relevance during adulthood.

Conclusions

Isolated TOF is a genetic disorder involving multiple genes. Although subjects show a range of individual mutations, the phenotypic outcome is similar because TOF genes show common patterns of functional interactions. Sequencing approaches can help to define a genetic risk profile for families that can also be used to define differences in the long-term clinical outcome, which should permit a personalization of diagnostic and therapeutic strategies.

S1.5 An Excess of Deleterious Variants in VEGF-A Pathway Genes in Down Syndrome-Associated Atrioventricular Septal Defects

Christine Ackerman[1], Adam E. Locke [2,8], Eleanor Feingold[3], Benjamin Reshey[1], Karina Espana[1], Janita Thusberg[4], Sean Mooney[4], Lora J. H. Bean[2], Kenneth J. Dooley[5], Clifford Cua[6], Roger H. Reeves[7], Stephanie L. Sherman[2], Cheryl L. Maslen[1,]*

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[4]Buck Institute for Research on Aging, Novato, CA 94945

[5]Sibley Heart Center Cardiology, Children's Hospital of Atlanta, Atlanta, GA 30033

[6]Heart Center, Nationwide Children's Hospital, Columbus, OH 43205

[7]Department of Physiology and the Institute for Genetic Medicine, Johns Hopkins University School of Medicine, Baltimore, MD 21205

[8]Current address: Center for Statistical Genetics, Department of Biostatistics, University of Michigan School of Public Health, Ann Arbor, MI 48109

About half of people with trisomy 21 have a congenital heart defect (CHD) while the remainder have a structurally normal heart, demonstrating that trisomy 21 is a significant risk factor but is not causal for abnormal heart development. We used a candidate gene approach in a study cohort of individuals with Down syndrome (DS) to determine if rare genetic variants in genes involved in atrioventricular valvuloseptal morphogenesis contribute to atrioventricular septal defects (AVSD) in this sensitized population. We found a significant excess (p<0.0001) of variants predicted to be deleterious in DS with AVSD cases compared to DS with no heart defect controls. The variants with the highest probability of being damaging were found in six genes: *COL6A1*, *COL6A2*, *CRELD1*, *FBLN2*, *FRZB* and *GATA5*. Several of the variants were recurrent in unrelated cases. There were no variants with an equal probablity of being damaging in these genes found in controls, demonstrating a highly specific association with AVSD. Of note, all of these genes are in the VEGF-A pathway even though the candidate genes analyzed in this study represented numerous biochemical and developmental pathways, suggesting that rare variants in the VEGF-A pathway may contribute to the genetic underpinnings of AVSD in humans.

S1.6 Identification of Novel Mutations in a Large Family with Different Forms of Congenital Heart Disease

AC Fahed, RC Tanos, † IG El-Rassy, † FF Bitar,† JG Seidman,* CE Seidman* , GM Nemer,†*
*Harvard Medical School, Boston, MA
†American University of Beirut, Beirut, Lebanon

Background: Congenital Heart Disease (CHD) affects more than 1% of newborns and is widely believed to be due to mutations in genes involved in cardiac development. However, to date only about 5% of the genetic causes of CHD is known.

Objective: Our objective is to study families with CHD from Lebanon, a highly consanguineous population, using a combination of Sanger and Next-Generation sequencing, as well as SNP genotyping.
Methods: Target-capture sequencing of cardiac-enriched genes was performed on more than 150 families with CHD. The phenotypes include septal defects (atrial and ventricular), valvular disease (pulmonary stenosis, aortic stenosis, tricuspid atresia, bicuspid aortic valve), coarctation of the aorta, tetralogy of fallot, transposition of the great arteries, single ventricle, Ebstein anomaly, and atrioventricular canal defects. Mutation-negative large multiplex families were studied using SNP genotyping and whole-exome sequencing.

Results: Target-capture sequencing analysis of 50 families identified several homozygous missense mutations in cardiac genes (JAG1, NOTCH1, MID1, NF1, NUP188, NFATc4, FLNA). Mutation segregation with the phenotype was confirmed in most of the families. Analysis of a particular large family with different forms of congenital diseases point out to a multi-factorial mode of inheritance involving Hand2, NFATC4, and Nephrin.

Conclusion: Dominant mutations occur in consanguineous populations. The combination of SNP genotyping and next-generation sequencing is an expected method to study gene mutations in families with congenital heart disease, particularly those with recessive mutations.

Platform Session 2: Cardiomyocyte Growth and Regeneration

S2.1 Cardiac hypertrophy in LEOPARD Syndrome is caused by *PTPN11* loss-of-function activity in the developing endocardium

Jessica Lauriol[1], Kimberly Keith[1], Boding Zhang[3], Roderick Bronson[2], Kyu-Ho Lee[3], and Maria I. Kontaridis[1,4]
[1]Department of Medicine, Division of Cardiology, Beth Israel Deaconess Medical Center, Boston, MA.
[2]Department of Pathology, Harvard Medical School, Boston, MA.
[3]Department of Cardiovascular Developmental Biology, Medical University of South Carolina, Charleston, SC.
[4]Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA.

Mutations in *PTPN11,* encoding the protein tyrosine phosphatase (PTP) SHP2, cause LEOPARD Syndrome (LS), an autosomal dominant disorder with multiple cardiac defects, including hypertrophy. Interestingly, LS mutations are catalytically inactive and behave as loss-of-function. However, how LS mutants affect cardiac development remains unclear.

We generated an inducible "knockin" mouse model expressing the LS-associated *Ptpn11* Y279C mutation (iLS/+). When crossed to EIIA deleter-Cre mice, these (now termed) LS/+ mice recapitulated nearly all aspects of the human LS phenotype, including hypertrophy. To determine whether defects in LS/+ mice could be attributed directly to aberrant cardiac developmental effects, we examined hearts from WT, LS/+ and LS/LS embryos at E10.5, E14.5, and E15.5. Both LS/+ and LS/LS developing hearts had significantly diminished trabeculation at E10.5, as well as valvular hyperplasia and ventricular septal defects (VSD) at E14.5, indicative of defective or delayed cardiac development. In addition, LS/LS embryos had persistent VSD at E15.5 and cardiac looping defects that resulted in dextraposition of the aorta, which led to embryonic lethality between E14.5 and E15.5.

To determine the lineage-specific effects, we generated neural crest (Wnt1::Cre)-, endothelial (Tie2::Cre)-, and myocardial (Nkx2.5::Cre)- specific LS/+ expressing mouse lines. Surprisingly, we found that only the endocardial-specific LS-expressing mice could completely recapitulate the embryonic cardiac developmental defects observed in the LS/+ phenotype. Moreover, these mice also developed the adult-onset hypertrophy, as observed in adult LS/+ mice. Taken together, our data indicate that the adult-onset cardiac hypertrophy associated with LS is caused by *PTPN11* loss-of-function effects that occur in the developing endocardium**.**

S2.2 Epigenetic regulation of cardiac hypertrophy

Qing-Jun Zhang[1], David Maloney[2], Anton Simeonov[2], and Zhi-Ping Liu[1]
[1]Department of Internal medicine, division of Cardiology, UT Southwestern
[2]National Institutes of Health Chemical Genomics Center, National Human Genome Research Institute.

One of the major challenges in managing and treating heart failure patients is to develop disease-modifying drugs that can prevent, reverse, or slow down the disease progression. Upon pathological insults, the heart undergoes remodeling processes, including left ventricular hypertrophy and reprogramming of gene expression. Understanding the mechanisms involved could provide a key to develop interventional therapeutics. Epigenetic modification of chromatin, including histone methylation, regulates gene transcription in response to environmental signals. JMJD2A is a trimethyl-lysine specific histone lysine demethylase. To study the role of JMJD2A, we generated heart specific JMJD2A overexpression and deletion mouse lines. Our studies with these genetically modified mice indicated that JMJD2A is required for pathological cardiac hypertrophy. Furthermore, we show that the demethylase activity of JMJD2A is required for its transcriptional activity. To test whether inhibition of JMJD2A enzymatic activity suppresses hypertrophic response, we identified several small molecule inhibitors of JMJD2A. These small molecule inhibitors of JMJD2A inhibited the phenylephrine-stimulated cardiomyocyte hypertrophy in vitro. Our data suggests that JMJD2A enzymatic activity may act as a hypertrophic determinant and may be an innovative drug target for prevention and treatment of pathological cardiac hypertrophy and heart failure.

S2.3 Regulation of striated muscle gene expression: A role for *Prox1* during cardiac and skeletal muscle development

Louisa K. Petchey[1], *Paul R. Riley*[1]
[1]Molecular Medicine Unit, UCL Institute of Child Health, London WC1N 1EH, UK

Isoform-specific expression of muscle structural genes is a crucial component of the structural, functional and metabolic distinction that exists between the fibre types comprising striated cardiac and skeletal muscle. The homeobox transcription factor *Prox1* has previously been shown to be expressed in slow-twitch skeletal muscle in zebrafish and to be required for normal cardiac muscle development in mice. Using *Nkx2.5-Cre*, *Myf5-Cre* and *Prox1*^{flox} mouse lines to knockout *Prox1* in both striated muscle types, we can identify for the first time a role for *Prox1* in the repression of three key fast-twitch skeletal muscle genes, *Tnnt3*, *Tnni2* and *Myl1*, in both cardiac and slow-twitch skeletal muscle. *Prox1* has an additional role in skeletal muscle in the regulation of myosin heavy chain isoform expression. This implicates *Prox1* in a complex regulatory network that determines fibre type in skeletal muscle, where it functions downstream of *Sox6* and upstream of *Six1*; and characterises a novel role for *Prox1* during heart development. The inappropriate expression of fast-twitch skeletal muscle genes is known to negatively impact cardiac function while some human cardiac and skeletal myopathies have been associated with incorrect isoform expression of structural genes. New opportunities for intervening in the molecular mechanisms that underlie these pathologies will be gained from a better understanding of their developmental regulation.

S2.4 CIP, a novel cardiac nuclear protein regulates cardiac function and remodeling

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Mammalian heart has minimal regenerative capacity. In response to mechanical or pathological stress, the heart undergoes cardiac remodeling. Pressure and volume overload in the heart cause increased size (hypertrophic growth) of cardiomyocytes. Whereas the regulatory pathways that activate cardiac hypertrophy have been well established, the molecular events that inhibit or repress cardiac hypertrophy are less known. Here, we report the identification, characterization and functional examination of CIP, a novel cardiac Isl1-interacting protein. CIP was identified from a bioinformatic search for novel cardiac-expressed genes in mouse embryonic hearts. CIP encodes a nuclear protein without recognizable motifs. Northern blotting, in situ hybridization and reporter gene tracing demonstrated that CIP is highly expressed in cardiomyocytes of developing and adult hearts. Yeast-two-hybrid screening identified Isl1, a LIM/homeodomain transcription factor essential for the specification of cardiac progenitor cells in the second heart field, as a co-factor of CIP. CIP directly interacted with Isl1 and we mapped the domains of these two proteins which mediate their interaction. We show that CIP represses the transcriptional activity of Isl1 in the activation of the MEF2C enhancer. The expression of CIP was dramatically reduced in hypertrophic cardiomyocytes. Most importantly, overexpression of CIP repressed agonist-induced cardiomyocyte hypertrophy. Interestingly, recent work showed that CIP also interacts with Lamin A/C (LMNA), a causative gene mutated in patients with DCM and other disorders. We found that the expression of CIP was markedly regulated in animal models of cardiac diseases, including HCM and DCM. Furthermore, our preliminary data showed that CIP knockout mice display impaired cardiac function. Together, our studies identify CIP a novel regulator of cardiac remodeling and disease.

S2.5 Yap1 regulates cardiomyocyte proliferation through PIK3CB

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Cardiomyocyte loss is a major underlying cause of heart failure. Works focusing on cardiomyocyte proliferation regulation have shown that adult cardiomyocytes do proliferate to a limited extent, but not enough to show any observable clinical benefits. Efforts are needed to deciphering genes capable of driving cardiomyocytes to re-enter cell cycle. The Hippo kinase cascade is an important regulator of organ growth, including heart. A major target of this kinase cascade is YAP1. Our data showed that fetal Yap1 inactivation caused marked, lethal myocardial hypoplasia and decreased cardiomyocyte proliferation, whereas fetal activation of YAP1 stimulated cardiomyocyte proliferation. Remarkably, YAP1 activation was sufficient to stimulate proliferation of postnatal cardiomyocytes, both in culture and in the intact heart. To characterize the mechanism of YAP1 induced cardiomyocyte proliferation, we carried out microarray and Chip sequencing assays. By combining the microarray da ta and the Chip sequencing results, we found several genes that possibly regulated by YAP1. PIK3CB was validated to be a direct target of Yap1, and a regulatory element residing in the first intron of PIK3CB was characterized. Knocking down of PIK3CB significantly reduced the YAP1 induced cardiomyocyte proliferation, however, TGX221, a specific PIK3CB kinase inhibitor failed to weaken the effects of YAP1 on cardiomyocyte proliferation. Overexpression of either PIK3CB, or PIK3CBK805R, a kinase dead mutation version, was sufficient to drive neonatal cardiomyocytes to re-enter cell cycle in vitro. These studies demonstrate that YAP1 is a crucial regulator of cardiomyocyte proliferation, and YAP1 regulates cardiomyocyte proliferation partially through PIK3CB.

S2.6 A Transitional Extracellular Matrix Instructs Epicardial-mediated Vertebrate Heart Regeneration

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Unlike humans, certain vertebrates including newts and zebrafish possess extraordinary abilities to functionally regenerate lost appendages and injured organs, including cardiac muscle. We aim to discover the underpinning mechanisms that regulate complex tissue rebuilding in these regeneration-competent species, ultimately developing strategies for regenerative wound healing in mammals. Here, we present new evidence that the extracellular matrix (ECM) provides signals to tissues and cells essential for the induction and maintenance of regenerative processes in cardiac muscle. Comprehensive mining of DNA microarrays of regenerating newt and zebrafish hearts with data comparison to the scar-forming response following myocardial infarction in mice and humans provided a signature of conserved regenerative gene activities. Differential expression and Gene Ontology analyses revealed that distinct ECM components and ECM-modifying proteases are among the earliest upregulated and most significantly enriched genes in response to injury in both the newt and zebrafish. In contrast, following mammalian cardiac injury, immune and inflammatory responses are significantly activated instead of ECM genes. Complementary immunohistochemistry studies in the newt demonstrated dynamic spatial and temporal changes in ECM composition between normal and regenerating heart tissues, especially in the epicardium early in the regenerative response. We show in vivo and under defined culture conditions that the proliferative and migratory response of cardiomyocytes is directly linked to distinct matrix remodeling in both the myocardium and epicardium of the regenerating newt heart. Collectively, these results provide a novel understanding of the regenerative process, suggesting that an evolutionarily conserved, regeneration-specific matrix instructs cell activities essential for cardiac muscle regeneration.

Platform Session 3: Genomics and Gene Regulation

S3.1 Isolation of nuclei from specific cardiac lineages in zebrafish for genome-wide and epigenomic analyses

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To understand the critical gene regulatory interactions that drive atrial versus ventricle identity, we are using genome-wide analysis of differentiating cardiomyocytes to uncover changes in epigenetic and transcriptome profiles. Although advances in sequencing technology have greatly enhanced our ability to characterize genome-wide changes in epigenetic modifications and gene expression, this has been constrained by the technical challenges faced in purifying specific cell lineages in adequate amounts from developing systems. Therefore we are developing a novel approach that will allow us to isolate nuclei of differentiating cardiomyocytes *in-vivo*.

We have created transgenic lines in which a biotin ligase recognition peptide (BLRP) is fused to an outer membrane nuclear envelope protein (NEP). Regulated expression of BirA enzyme drives the *in vivo* biotinylation of BLRP-NEP, allowing purification of nuclei with streptavidin-conjugated beads. When BirA is ubiquitously expressed, we can successfully elute chromatin threefold higher from transgenic embryos with biotinylated nuclei than transgenic embryos devoid of BirA enzyme. We successfully performed ChIP with multiple histone marks, indicating that the chromatin within these purified nuclei is useful for epigenomic analyses. We are creating double transgenics in which BLRP-NEP is biotinylated in specific heart lineages in order to capture chromatin and nuclear RNA (mRNA and miRNA) from cardiac progenitors, ventricle precursors and atrial precursors. This will allow us to perform genome-wide analysis of epigenetic and transciptome profiles in specific subsets of lineages and tissues during any stage of zebrafish cardiac development.

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S3.2 TU-tagging in mice defines dynamic gene expression programs in specific cardiovascular or other cell types within intact tissues

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Changes in gene expression programs underlie all aspects of cardiovascular development and disease. Therefore, transcriptional profiling is a powerful and direct approach to study these and other biological processes. Current methods are limited by difficulties isolating specific cell populations from complex tissues, changes in gene expression that occur during sample preparation, and the inability to distinguish dynamic transcriptional changes within a cellular pool of pre-existing RNA. To overcome these limitations in mouse research, we have developed a method called “TU-tagging” that allows for purification of newly transcribed RNA from defined cell types. With TU-tagging, genetic and chemical methods intersect to provide spatiotemporal controlled RNA labeling in vivo. Cre-induced expression of UPRT using a newly-developed transgenic mouse (*CA:loxStoploxUPRT*) directs the incorporation of injected 4-thiouracil (4TU) into nascent RNA, generating a pulse of cell type-specific thiolated RNA. The thio-RNA is readily purified from whole tissue RNA preparations and RNA-seq is used to define “acute” transcriptomes. We validate mouse TU-tagging by using *Tie2:Cre;CA:loxStoploxUPRT* double transgenic mice to purify endothelial RNA from brain or endothelial/endocardial RNA from heart at both embryonic and postnatal stages. In each case, the vast majority of the most enriched transcripts are verified as being expressed in *Tie2:Cre* marked cell lineages. We define a core set of pan-endothelial transcripts and identify groups of endothelial/endocardial lineage transcripts with shared specific expression patterns. TU-tagging provides a novel, sensitive method for characterizing transcriptome dynamics in rare cell types within the intact mouse.

S3.3 Escape of Gene Body DNA Methylation in Cardiac Genes is Cooperative with the Cell Type-specific Expression Patterns

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Mammalian genome is highly methylated in C-G dinucleotides (CpGs), over 60% of which are believed to be methylated. The effect of DNA methylation has been investigated intensively in the promoter regions, but it has still been unknown in non-promoter regions, especially in the cell type-specific effect. Although we previously reported that gene body DNA methylation levels are correlated with the transcription levels and the replication timing in human cell lines (Suzuki et al. Genome Res 2011), function of gene body DNA methylation levels in developing cells is unknown. In this study, we focused on the DNA methylation pattern in cardiomyocytes as the established differentiated cells. We examined genome-wide DNA methylation profiles of cardiomyocytes and non-cardiomyocyte cells from different developmental stages by the deep sequencing technique, and found that the gene body regions of some cardiac genes, including myosin heavy chain (MHC) genes, are less methylated than other genes, where the DNA methylation levels were dramatically decreased in the latter half of pregnancy. To evaluate the effect of gene body DNA methylation, we performed luciferase reporter assay by the reconstruction using the SssI-methylated and unmethylated gene body fragments, and confirmed that the gene body DNA methylation is repressive in the gene expression, mostly at the transcriptional level. Collectively, the gene body DNA methylation levels in some cardiac genes were developmentally regulated and possibly enhance the transcription of cardiac genes.

S3.4 The novel mechanism of histone-chromatin regulation for cardiomyocytes regeneration

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Fishes and amphibians have high capacities for heart regeneration even in the adult, whereas we mammalians lose such regeneration capacities. Understanding the mechanism of heart regeneration should provide the most important cues on the therapies for human heart failure. We found that SWI/SNF-BAF type cardiac chromatin remodeling factors and histone regulators were strongly up-regulated within 12 hours after resection of ventricle both in neonatal mice and axolotl, and these expression was maintained for one week during regeneration. To address whether these factors function as the early response factors in mammalian heart regeneration, we constructed BAF-overexpression (BAF-TG) mice, which showed stable expression of BAFs even in the adult heart. Interestingly, BAF-TG prevented fibrosis post myocardial infarction and regenerated their injured parts with the proliferation of cardiomyocytes. In vivo ChIP analyses revealed that the SWI/SNF core factor, Brg1, directly regulated several cardiac fetal genes' promoters such as Nppa, Tnnt2, Myl7 only at the embryonic stages. Surprisingly, in the BAF-TG, major cardiac fetal genes' promoters were still opened in the adult state. And si-treatment of polycomb or repress-type chromatin factor s increased cell proliferation in vitro. These data demonstrate that histone-chromatin conformation is changed depending on the developmental stages on several cardiac genes relating with the regeneration capacity.

S3.5 Epigenetic regionalization of the developing mouse heart

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A multitude of differences in gene expression have been described and correlated with cardiac chamber identity, structure, function and disease. Nonetheless, it is not fully resolved how these transcriptional spatial asymmetries, evident at even the very early stages of morphogenesis, are established and maintained. An intriguing hypothesis is that these asymmetries are contributed to by differences in chromatin accessibility, which potentiate or exclude expression of specific genes. By performing transcriptional profiling by RNA-seq on isolated left and right ventricles of embryonic mice and correlating the findings with ventricular ChIP-seq data for H3K27me3, a mark of closed chromatin associated with gene repression, and H3K4me1, a histone modification associated with open chromatin and active genes, we observe a significant subset of chamber-specific cardiac genes, including Tbx5, that are selectively marked by both H3K4me1 and H3K27me3 in opposite chambers. In the left ventricle, these genes are functionally linked to roles in energy metabolism, whereas in the right ventricle, coordinately marked genes are associated with developmental roles. We demonstrate that Tbx5 enhancers have the potential of driving expression in broad cardiac domains, and that the restricted Tbx5 expression is mainly due to regionalization of epigenetic marks. Furthermore, this spatially regionalized pattern of gene repression is redeployed to limit Tbx5 expression to forelimb and not hindlimb, highlighting a general chromatin-based mechanism to affect spatially-restricted expression. Beyond our general understanding of how the developing heart is patterned, these findings have implications for efforts at directed differentiation of stem cells into cardiomyocytes for therapeutic uses.

S3.6 *Drosophila* microRNA-1 Genetically Interacts with *little imaginal discs*, a histone demethylase.

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The expression pattern and function for the microRNA *miR-1* is conserved in both invertebrates and vertebrates, suggesting that it modulates some of the most fundamental processes within cardiac and skeletal muscle, including Notch signaling. We performed a forward genetic screen in *Drosophila* that identified *little imaginal discs* (*lid*), a chromatin-remodeling enzyme, as a genetic partner of *dmiR-1*. Lid demethylates histone H3 lysine 4 (H3K4), thereby epigenetically decreasing the activity of selected promotors. Lid forms a complex with Notch pathway regulatory proteins, and loss of lid activity results in de-repression of Notch downstream targets. We have found that in flies, heterozygosity of *lid* in the context of *dmiR-1* overexpression resulted in exacerbation of the *dmiR-1* overexpression phenotype, consistent with de-repression of Notch signaling. Furthermore, we discovered that use of a balancer chromosome containing a mutation in the Notch ligand *Serrate*, unmasked an indirect flight muscle phenotype in $lid^{(-/-)}$ flies that was modified upon manipulation of *dmiR-1* levels. These results imply that *dmiR-1* may selectively modulate the epigenetic modification of histones at Notch-responsive loci during fly heart and muscle development.

Platform Session 4: Neural Crest and Vascular Development

S4.1 Early neural crest-restricted Noggin over-expression results in congenital craniofacial and cardiovascular defects

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Noggin is a secreted BMP antagonist that binds BMP ligands and prevents their activation of receptors. Noggin (in concert with Wnts, FGFs, Delta/Notch) plays a key upstream role in neural crest cell (NCC) induction by generating a BMP morphogenic gradient that activates a cascade of transcription factors culminating in the ultimate steps of NCC differentiation. NCC derivatives are absolutely essential for cardiac outflow tract (OFT) remodeling, specifically during OFT septation and patterning of the great vessels that exit the heart.

Using Cre/loxP, constitutive Noggin expression was induced within NCC via *Pax3^{Cre}* (very early within neural tube), *Wnt1-Cre* (early within neural tube) or *Peri-Cre* (post-migratory) drivers. Consistent with its *in vitro* role in negatively regulating BMP signaling, Westerns confirmed *Noggin* overexpression significantly suppressed phosphorylation levels of Smad1/5/8 but left Smad2/3 unaffected *in vivo*. Moreover, *Wnt1-Cre* and *Pax3^{Cre}*-mediated suppression of BMP signaling resulted in subsequent loss of NCC-derived craniofacial, pharyngeal and OFT cushion tissues. Increased cell death was observed in pharyngeal arch NCC and DRG (although cell proliferation was unaltered), but not within the OFT itself. Lineage mapping demonstrated that NCC emigration was not affected in either *Pax3^{Cre};Noggin* or *Wnt1-Cre;Noggin* mutants, but that subsequent colonization of the OFT was significantly reduced in *Wnt1-Cre;Noggin* and completely absent in *Pax3^{Cre};Noggin* mutants. Further, although *Wnt1-Cre;Noggin* mutants are viable until birth, *Pax3^{Cre};Noggin* mutants all die by E14. Conversely, *Peri-Cre* mediated suppression of BMP signaling in post-migratory NCC had no effect and mutants are viable at birth. Taken together, these data show that conditional BMP inhibition within the NCC may exhibit different effects dependent upon timing and that tightly regulated TGFβ superfamily signaling plays an essential role during craniofacial and cardiac NCC survival *in vivo*.

S4.2 The Role of Fibronectin - Integrin Signaling in Pharyngeal Microenvironment in Modulation of Cardiac Neural Crest Cell Development

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Patterning of Pharyngeal Arch Arteries (PAAs) is a critical process in embryonic cardiovascular development. Alterations that perturb this process cause cardiovascular abnormalities such as human DiGeorge Syndrome. Our lab previously discovered defective development of CNCCs in global fibronectin (FN)-null or integrin a5-null mutants. In order to determine the roles of FN synthesized by the pharyngeal microenvironment during CNCC development, we conditionally ablated FN or the α5 subunit of its integrin receptor using *Isl1*^{Cre} strain of mice. We found that conditional inactivation of FN or integrinα5 using *Isl1*^{Cre} cause embryonic/neonatal lethality. Both of these conditional mutants display various cardiovascular and glandular anomalies associated with defective PAA and CNCC development. Moreover, histological analyses reveal defective development of ventricular myocardium and muscular ventricular septum, phenotypes dependent on the proper development of the mesodermal cardiac progenitors. Taken together, these phenotypes suggest that FN-integrin α5 signaling to distinct tissues comprising pharyngeal microenvironment play critical roles in PAA development by modulating interactions between CNCCs and non-CNCCs. To understand the basis of these phenotypes, we have investigated the contribution of CNCC to PAA formation/remodeling and found that FN promotes survival of CNCCs and cardiac progenitors. In addition, FN synthesized by pharyngeal tissues regulates CNCC navigation. We are investigating possible roles of FN and integrin α5-regulated signaling in regulation of morphogens, guidance molecules and growth factor signaling to tissues comprising the pharyngeal microenvironment. Taken together, our studies provide important insights into how tissue microenvironment regulates morphogenesis of complex organs from diverse populations of progenitors.

S4.3 Slit3-Robo1/2 signalling controls cardiac innervation and ventricular septum development by regulating neural crest cell survival

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The Slit-Robo signalling pathway has been shown to have pleiotropic effects in Drosophila heart development, however, its involvement in mammalian heart formation is yet largely unknown. Here, we analysed the role of this signalling pathway during murine heart development. We observed extensive expression of both Robo1 and Robo2 receptors and their ligands, Slit2 and Slit3, in and around the developing heart. Analysis of mice lacking Robo1 revealed membranous ventricular septum defects and decreased cardiac innervation, while animals lacking Robo2 did not display such defects. However, the combined absence of both Robo1 and Robo2 caused increased incidence and severity of membranous ventricular septum defects. Mice lacking the Slit1 and Slit2 ligands did not reveal any abnormalities, but Slit3 mutants recapitulated the membranous ventricular septum defect. Interestingly, the density of cardiac innervation was increased in Slit3 mutants. The combined absence of the membranous ventricular septum and innervation defects suggested a defect in cardiac neural crest contribution. Detailed cell counts showed a reduction in neural crest cell contribution to the outflow tract. Further analyses indicated increased apoptosis in the neural crest, just before entering the heart, suggesting that cell death underlies its reduced contribution to the heart. Our data indicate a novel role for Slit3-Robo1/2 interaction in cardiac neural crest survival and, thereby, in the formation of the membranous ventricular septum and cardiac innervation.

S4.4 The neural crest contributes to coronary artery smooth muscle formation through endothelin signaling

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Endothelin-1 (ET1) /Endothelin A receptor (ETAR) axis has important roles in regulating cardiovascular homeostasis and organ development. We have previously reported that ET1 and ETAR knockout mice display craniofacial defects and aortic arch malformations. We also found their expressions in the developing coronary artery smooth muscle cells (CASMCs), however their roles in coronary artery development had been unknown. Here we show that ET1/ETAR axis insufficiency disturbs coronary artery development. Fetal coronary angiography revealed some septal branches were abnormally enlarged in both ET-1 and ETAR KO mice at embryonic day 17.5 (E17.5) and the branching pattern of the septal branch was also altered in both KO mice. Immunohistochemical analysis revealed partial lack of CASMCs in KO mice and serial analysis showed these malformations were occurred at the remodeling stage of coronary arteries. Each phenotype appeared in the septal branch-specific manner, then we hypothesized these segment specificities stood on the variety of cell sources of CASMCs. Fate mapping using Wnt1-Cre mice suggested that lacked CASMCs were specifically derived from the neural crest cells.

To analyze the contribution of neural crest cells to CASMCs in detail, we used quail-chick chimera technique and neural crest ablation models. Quail-chick chimera with a particular region of the neural crest recaptured the preferential distribution pattern to the interventricular septum region. Chick ablation models also showed coronary artery malformations similar to those in ET-1 and ETAR KO mice. These results indicate novel contribution of the neural crest to coronary artery formation partly through endothelin signaling.

S4.5 CASTOR directly regulates a novel Egfl7/RhoA pathway to promote blood vessel development and morphogenesis

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The formation of the vascular system is essential for embryonic development and homeostasis. Consistent with recent genome wide association studies implicating a genetic link between the transcription factor CASTOR (CST) and high blood pressure and hypertension, we demonstrate here that CST has an evolutionarily conserved function in blood vessel formation; in the absence of CST, Xenopus embryos fail to develop a fully branched and lumenized vascular system and human endothelial cells (HUVECs) lacking CST display dramatic defects in adhesion/contractility and cell division. Using chromatin immunoprecipitation (ChIP), we go on to identify Epidermal growth factor-like domain 7 (Egfl7) as a direct transcriptional target of CST. We demonstrate that CST is required for expression of Egfl7, that CST is endogenously bound to the Egfl7 locus in the developing embryo, and that depletion of EGFL7 phenocopies CST depletion in whole embryos and in HUVECs. We further show that the adhesio n/contractility abnormalities due to depletion of CST in HUVECs can be rescued by the reintroduction of EGFL7. Critically, we have demonstrated that CST and EGFL7 modulate endothelial cell shape and contractility through the small GTPase RhoA. Collectively, these data support a mechanism whereby CST directly regulates its transcriptional target Egfl7 to promote RhoA-mediated endothelial cell shape and adhesion changes to establish the vasculature and hence, these studies provide insight into the cellular and molecular basis for vascular disease associated with the loss of CST.

S4.6 Nephronectin regulates axial vein morphogenesis in zebrafish

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Angiogenesis is the development of new vessels from pre-existing vessels. This is a critical morphological event both in organ development as well as in diseases. Like in other vertebrates, in zebrafish vessels form a complex network in order to fulfill tissue oxygen demands. Development of complex vascular networks is dependent on the directional migration of groups of endothelial cells, which is called angiogenic sprouting. Here we have demonstrated that in zebrafish the extracellular matrix protein, nephronectin, is transiently expressed in the caudal vein plexus forming region at the time of caudal vein sprouting at around 30 hours post fertilization (hpf). Morpholino-mediated nephronectin depletion resulted in the malformation of the caudal vein plexus and the ventral vein and in the frequent loss of inter-segmental veins. Time-lapse analysis from 28 hpf to 40 hpf indicated a decreased in the frequency of caudal vein sprout formation in nephronectin morphants. In addition, existing sprouting appeared multi-directional indicating a navigation problem. Biochemical analysis demonstrated that nephronectin is able to bind to the integrin αV/β3 heterodimer. Importantly, integrin αV and nephronectin expression overlapped in the region of the caudal vein plexus. Moreover, morpholino-mediated integrin αV knockdown in zebrafish phenocopied nephronectin depletion. Taken together, our data indicate that nephronectin regulates directional sprouting of the axial vein in zebrafish, which might be via integrin αV.

Platform Session 5: Coronaries, Epicardium, and Conduction System

S5.1 Ets-1 Regulates the Migration of Coronary Vascular Precurors and Coronary Endothelial Cell Proliferation

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We have recently described a cardiac developmental defect in mice deficient in the transcription factor Ets-1. These mice die in the perinatal period with ventricular septal defects and an ectopic focus of cartilage in their outflow tract myocardium. We demonstrated that these defects are the result of altered migration and differentiation of the cardiac neural crest due to the loss of Ets-1. In this report, we describe an additional role for Ets-1 during cardiac development. We observed left ventricular systolic dysfunction in Ets-$1^{-/-}$ mice as measured by a decrease in fractional shortening (45.3% vs. 22.5%, $p<0.0001$). In addition, we found a 36% reduction in myocardial capillary density in newborn Ets-1 deficient mice, consistent with the notion that the left ventricular dysfunction seen in Ets-$1^{-/-}$ mice is due to an inadequately developed myocardial capillary bed. To examine earlier steps in coronary vasculogenesis, we performed whole-mount PCAM staining of E12.5 to E14.5 Ets-$1^{-/-}$ hearts and found significant attenuation in the development of the coronary vascular plexus. The appearance of both arterial and venous coronary precursors in the subepicardium was delayed, suggesting a global defect in the migration of these cells. In addition, phospho-histone H3 immunohistochemistry demonstrated a 41% reduction in the proliferation of endothelial cells in Ets-$1^{-/-}$ hearts at E16.5. Taken together, these results suggest that Ets-1 plays an important role in both the migration and proliferation of coronary endothelial cells during cardiac development.

S5.2 Wnt signaling in the developing murine epicardium and epicardium-derived-cells (EPDCs)

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The epicardium is the outermost layer of the heart and develops from a mesothelial cluster of cells at its venous pole (the proepicardium). A subset of these cells invades the underlying subepicardium and myocardium via an epithelial-to-mesenchymal-transition (EMT). Besides this cellular contribution additional epicardium-derived paracrine signals are important for myocardial and coronary vessel development. Previous work suggested a requirement of canonical Wnt signaling in the epicardium for EMT and coronary smooth muscle cell development. Here, we reinvestigated the role of canonical Wnt signaling in the epicardium and epicardium-derived-cells during embryogenesis, using an alternative conditional genetic approach. In the background of a Tbx18cre-line showing recombination in the proepicardium and its derivatives, ß-catenin (Ctnnb1) loss- and gain-of-function alleles were analyzed. Surprisingly, mice deficient for Ctnnb1 are live born and show neither impaired coronary artery formation, nor a defective epicardium. The ability of epicardium-derived explants to differentiate into the smooth muscle lineage is maintained. Stabilization of Ctnnb1 in the epicardium resulted in lethality between embryonic days eleven and twelve. Further lineage analysis revealed the formation of undifferentiated cell aggregates on the surface of the developing heart and impaired EMT.
Our data contradicts previous work, by demonstrating that canonical Wnt signaling in epicardium and EPDCs is dispensable for coronary artery formation in the developing embryo. Moreover constitutive active Wnt signaling in these cells leads to early lethality, indicating only a minor physiological role of Wnt signaling in the epicardium and epicarium-derived-cells during development.

S5.3 Determination of Cardiac Pacemaker Cell Origins Reveals a Critical Role for Wnt Signaling During their Cell Fate Specification.

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Cardiac pacemaker cells have unique molecular and physiological characteristics that allow them to rapidly and autonomously generate action potentials. How and when pacemaker cell fate diversifies from adjacent myocardial cell types and are programmed to attain these specialized features, however, remains poorly understood. Here we report our fate mapping studies demonstrating that just following gastrulation pacemaker progenitors reside in the right lateral plate mesoderm posterior to the known heart fields. Our data further indicate that this pacemaker precursor region overlaps with the expression of at least one canonical Wnt, Wnt8c. This is surprising as canonical Wnts are thought to be inhibitory for myocardial specification during these stages. In order to test whether Wnt signaling is required to specifically induce pacemaker versus working myocardial fate, we injected cells expressing the soluble Wnt antagonists, Crescent, directly into the pacemaker region of gast rulating embryos. Ectopic Crescent resulted in both the expansion of the transcription factor NKX2.5 and the down-regulation of at least one pacemaker ion channel, HCN4, within the pacemaker precursors. Conversely, introduction of canonical Wnts into the heart field mesoderm was capable of inducing ectopic pacing sites and the formation of NKX2.5 negative HCN4 positive myocytes within the looping heart tube, suggesting a conversion of working myocytes into pacemaker-like cells. These data demonstrate that pacemaker progenitor specification depend on signaling cues unique from working myocardium and that Wnt signaling, at least in part, supports divergence into the pacemaker lineage. This work is supported in part by grants from the NIH-NLHBI.

S5.4 A Tbx5-Scn5a Molecular Network Modulates Function of the Cardiac Conduction System

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Cardiac conduction system (CCS) disease is common with significant morbidity and mortality. Current treatment options are limited and rational efforts to develop cell-based and regenerative therapies require knowledge of the molecular networks that establish and maintain CCS function. Emerging evidence points to key CCS roles for genes with established roles in heart development. Recent genome wide association studies (GWAS) have identified numerous loci associated with human CCS function including TBX5 and SCN5A. We hypothesized that Tbx5, a critical developmental transcription factor, regulates networks required for mature CCS function. Removal of Tbx5 from the mature VCS resulted in severe VCS functional consequences, including loss of fast conduction, arrhythmias, and sudden death. Ventricular contractile function and the VCS fate map remained unchanged in VCS-specific Tbx5 knockouts. However, key mediators of fast conduction including Cx40 and Nav1.5, encoded by Scn5a, demonstrated Tbx5-dependent expression in the VCS. We identified a Tbx5-responsive enhancer downstream of Scn5a sufficient to drive VCS expression in vivo, dependent on canonical T-box binding sites. Our results establish a direct molecular link between Tbx5 and Scn5a and establish a hierarchy between human GWAS loci that affects VCS function in the mature CCS, establishing a paradigm for understanding the molecular pathology of VCS disease. Ongoing studies to dissect the regulation of Scn5a expression using a Scn5a-LacZ BAC transgenic reporter system have identified two Scn5 a enhancers sufficient for establishing the native Scn5a expression pattern. Implications for the role of Scn5a and Scn10a in conduction system function, variability, and arrhythmia susceptibility will be discussed.

S5.5 Mouse cardiac T-box targets reveal an Scn5a/10a enhancer functionally affected by genetic variation in humans

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The contraction pattern of the heart relies on the activation and conduction of the electrical impulse. Perturbations of cardiac conduction have been associated with congenital and acquired arrhythmias and cardiac arrest. The pattern of conduction depends on the regulation of heterogeneous gene expression by key transcription factors and transcriptional enhancers. Here, we assessed the genome-wide occupation of conduction system regulating transcription factors Tbx3, Nkx2-5 and Gata4 and of enhancer-associated co-activator p300 in the mouse heart, uncovering cardiac enhancers throughout the genome. Many of the enhancers co-localize with ion channel genes repressed by Tbx3, including the clustered sodium channel genes Scn5a, essential for cardiac function, and Scn10a. We identify two enhancers in the Scn5a/Scn10a locus, which are regulated by Tbx3 and its family member and activator Tbx5, and are functionally conserved in humans. We provide evidence that a single-nucleotide po lymorphism in the SCN10A enhancer, associated with alterations in cardiac conduction patterns in humans, disrupts Tbx3/5 binding and reduces the cardiac activity of the enhancer in vivo. Thus, the identification of key regulatory elements for cardiac conduction helps to explain how genetic variants in non-coding regulatory DNA sequences influence the regulation of cardiac conduction and the predisposition for cardiac arrhythmias.

S5.6 Activation of voltage-dependent anion channel 2 suppresses Ca^{2+}-induced cardiac arrhythmia

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While a role of Ca^{2+} in establishing embryonic cardiac rhythmicity has been proposed, the underlying mechanisms have yet to be fully explored. The zebrafish *tremblor* (*tre*) mutant embryo lacks functional cardiac Na^{+}/Ca^{2+} exchanger, resulting in Ca^{2+} extrusion defects, abnormal Ca^{2+} transients and unsynchronized cardiac contractions. We therefore use this mutant as an animal model to dissect the molecular network essential for controlling Ca^{2+} homeostasis in the heart. From a chemical suppressor screen, we identified a synthetic compound named efsevin, which effectively restores synchronized cardiac contractions in *tre* embryos. In addition, efsevin suppresses arrhythmogenic Ca^{2+} waves by accelerating the decay phase of Ca^{2+} sparks in murine cardiomyocytes, demonstrating that efsevin modulates cardiac Ca^{2+} handling. Through a biochemical pull-down assay, we identified a direct interaction between efsevin and the mitochondrial outer membrane protein VDAC2. Overexpression of VDAC2 restores synchronized cardiac contractions in *tre* embryos and knock-down of VDAC2 attenuates the rescue effect of efsevin, indicating that efsevin modulates Ca^{2+} handling by enhancing the activity of VDAC2. Furthermore, overexpression of mitochondrial inner membrane Ca^{2+} uptake proteins restores cardiac contractions in *tre* embryos. This effect is further enhanced when VDAC2 is co-expressed, and is abolished in *tre/vdac2* double deficient embryos. Taken together, our data reveal an essential role for mitochondria in regulating cardiac Ca^{2+} homeostasis and rhythmicity. Our findings also establish VDAC2 as a critical gate for mitochondrial Ca^{2+} uptake and efsevin as a potential therapeutic agent for cardiac arrhythmia.

Platform Session 6: Early Heart Formation and Progenitors

S6.1 Quantitative analysis of polarity in 3D reveals local cell coordination in the embryonic mouse heart

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Several mutations in genes encoding members of the Planar Cell Polarity pathways impair heart morphogenesis. However, no asymmetric localization of such proteins has been reported in myocardial cells and it has remained unclear how the embryonic myocardium is polarized. We have now identified cell polarity markers in the mouse and aim to quantify the degree of coordination between myocardial cells in the embryonic heart. Classically, anisotropies which underlie organ morphogenesis have been quantified as a 2D problem, taking advantage of a reference axis. However, this is not applicable to the looped heart tube, which has a complex geometry. We have designed a 3D image processing framework, to map the regions in which cell polarities are significantly aligned. This novel procedure integrates multidisciplinary tools, including image segmentation, statistical analyses, axial clustering and correlation analysis. The result is a sensitive and unbiased assessment of the significant alignment of cell orientations in 3D, compared to a random axial distribution. We show that the axes of cell polarity, defined as the centrosome-nucleus axes, are frequently biased in a plane parallel to the outer surface of the heart, with a minor transmural component. This reflects the expansion of the cardiac chambers which precedes transmural thickening. Our study reveals that ventricular cells locally coordinate their axes over 100µm. This is in keeping with the growth of the myocardium, that we had shown by clonal analysis to be regionally oriented.

S6.2 Clonally dominant cardiomyocytes direct heart morphogenesis

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As vertebrate embryos develop into adulthood, their organs dramatically increase in size and change tissue architecture. Here, we used a multicolor clonal analysis to define the contributions of many individual cardiomyocytes as the zebrafish heart undergoes morphogenesis from a primitive embryonic structure into its complex adult form. We find that the single cardiomyocyte-thick wall of the juvenile ventricle forms by lateral expansion of several dozen cardiomyocytes into muscle patches of variable sizes and shapes. As juveniles mature into adults, this structure becomes fully enveloped by a new lineage of cortical muscle. Adult cortical muscle originates from a small number (~8) of cardiomyocytes that display clonal dominance reminiscent of stem cell populations. Cortical cardiomyocytes initially emerge from internal myofibers that in rare events breach the juvenile ventricular wall and expand over the surface. Our study illuminates dynamic proliferative behaviors that generate adult cardiac structure, revealing clonal dominance as a key mechanism that shapes a vertebrate organ.

S6.3 $G\alpha_{13}$ is required for S1pr2-mediated myocardial migration by regulating endoderm morphogenesis

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During vertebrate development, bilateral populations of myocardial precursors migrate towards the midline to form the primitive heart tube, and this requires both their intrinsic properties and the appropriate environment, including endoderm. Disruption of myocardial migration leads to the formation of two bilaterally located hearts, a condition known as cardiac bifida. In zebrafish, signaling mediated by sphingosine-1-phosphate (S1P) and its cognate G protein-coupled receptor (S1pr2/Mil) controls myocardial migration. However, the underlying downstream mechanism remains poorly understood. Here we show that depletion of specifically the G protein isoform $G\alpha_{13}$, results in cardia bifida and tail blistering, defects reminiscent of those observed in embryos deficient for S1P signaling. Our genetic studies indicate that $G\alpha_{13}$ acts downstream of S1pr2 to regulate myocardial migration through a RhoGEF/Rho-dependent pathway. Intriguingly, both cardiomyocytes and endoderm express S1pr2 and $G\alpha_{13}$, and cardiac-specific expression of $G\alpha_{13}$ fails to rescue cardia bifida in the context of global $G\alpha_{13}$ inhibition. Furthermore, we found that disruption of any component of the S1pr2/$G\alpha_{13}$/RhoGEF signaling pathway led to defects in endoderm morphogenesis that were correlated to cardia bifida, and that defects in both endoderm and myocardial migration resulting from $G\alpha_{13}$ depletion were rescued coincidently. Overall, our findings represent the first evidence that S1pr2 controls myocardial migration through a $G\alpha_{13}$/RhoGEF-dependent pathway, and that it does so by regulating endoderm morphogenesis. Currently, we are investigating the mechanisms by which S1pr2/$G\alpha_{13}$ signaling controls endoderm morphogenesis.

S6.4 A Cdc42 associated genetic network directs heart lumen formation and morphogenesis in Drosophila

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The *Drosophila* embryonic heart is a key model system for understanding heart specification. Our previous studies indicate that heart morphogenesis requires Slit/Robo signaling, a function conserved in vertebrates. The mechanisms by which these and other signals control heart formation are still unknown. Due to its role in membrane dynamics, we investigated the role of the small GTPase Cdc42 during *Drosophila* heart development and found it to be required for cardiac cell alignment and heart tube formation. Mutant or constitutively active Cdc42 in the developing heart causes improper cardioblast alignment and formation of multiple lumina, suggesting that Cdc42 is required during discrete steps of cardiogenesis. Cell polarity and filopodia dynamics are unaffected by loss of Cdc42, therefore Cdc42 might have a different role during heart morphogenesis. To understand the regulation of Cdc42 and to identify new genetic interactors, we performed a genetic screen for modifiers of Cdc42. We identified the tyrosine kinase Abelson (Abl), and the non-muscle myosin-II zipper to strongly interact with Cdc42. Abl itself shows a requirement for coordinated heart tube assembly, and Zipper exhibits a dynamic localization pattern during cardiogenesis, which depends on Cdc42 function, but is independent of Slit/Robo. Activation of the formin-like protein Diaphanous (Dia) produced defects similar to activated Cdc42, indicating that control of cell shape changes is a key regulatory step during heart morphogenesis. Our data suggest a novel mechanism of cardiac morphogenesis involving Abl, Cdc42, Dia and Zip acting in a common pathway during cardiac cell shape changes and orchestrated heart lumen formation.

S6.5 Identification and characterization of a multipotent cardiac precursor

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Construction of the vertebrate heart is a complex process involving the regulated contribution of cardiac progenitor cells in a discrete spatial and temporal order. The precision required for this process is highlighted by the frequency of congenital heart defects. Understanding the identity and regulation of these progenitors is critical to understanding the origins of congenital heart defects and has the potential to lead to novel cell-based regenerative therapies for heart disease. Previous lineage tracing studies have predicted the existence of an early, multi-potent cardiovascular progenitor, but the identity of this progenitor has remained undefined. The transcription factor *Mesp1* has been postulated to mark early cardiac progenitors, however our lineage tracing results suggest that *Mesp1* labels a much broader domain of cardiac and non-cardiac mesoderm than initially reported. *Baf60c/Smarcd3*, a subunit of the BAF chromatin-remodeling complex, is expressed specifically in the heart and somites in the early mouse embryo. We have identified an early domain of *Smarcd3* expression, prior to formation of the cardiac crescent in a region of the late gastrula stage mouse embryo predicted to contain a cardiovascular progenitor population. Temporally-regulated lineage tracing of this population specifically labels both first and second heart field derived structures, including the endocardium, myocardium, and epicardium, and a population of cells within the anterior forelimb. Ongoing work will describe the precise temporal and spatial localization of *Smarcd3* relative to other markers of early cardiogenesis (*Tbx5*, *Isl1*, and *Nkx2-5*) as well as the clonal relationship among these early *Smarcd3*-expressing cells.

S6.6 Distinct origin and commitment of HCN4+ First Heart Field cells towards cardiomyogenic cell lineages

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Most of the mammalian heart is formed from two anatomically and molecularly distinct groups of cells of mesodermal origin termed the first and second heart fields (FHF and SHF). Whereas the SHF gives rise to the right ventricle, parts of the atria, and proximal region of the outflow-tract, the FHF gives rise to the left ventricle and remaining parts of the atria. Multipotent progenitors of the SHF are marked preferentially by Islet-1 expression, while a marker exclusive to FHF progenitors has not been identified. Here, we present Hcn4, a gene encoding the hyperpolarization-activated cyclic nucleotide-gated channel 4, as a new FHF marker expressed as early as pre-crescent stage. In-situ hybridization analysis revealed a dynamic expression of Hcn4 in the developing heart, with its strongest expression in the cardiac crescent and down-regulation by E9.5. HCN4-CreErt2 lineage tracing experiments confirm that the progeny of these cells give rise to FHF-derived structures of the heart. Surprisingly, these cells contribute primarily to cardiomyogenic cell lineages, suggesting that Hcn4+ FHF precursor cells are committed cardiomyogenic progenitors from their earliest detectable stage. Single cell clonal analysis using Hcn4+ FHF cells from both mouse embryos and differentiated mouse embryonic stem cells support this finding.
Our data suggests a model of cardiogenesis, where the primary purpose of the FHF is to generate cardiac muscle to support the contractile activity of the primitive heart tube, whereas the contribution of SHF-derived progenitors to the heart is more diverse, leading to cardiomyocytes, smooth muscle and endothelial cells.

Platform Session 7: Chamber Development

S7.1 *irx1a* Acts Downstream of *nkx* Genes in Maintaining Cardiac Chamber Identity in Zebrafish

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Maintenance of the features characteristic of each cardiac chamber is essential to ensure efficient and organized contractility of the heart. Despite the importance of preserving chamber-specific characteristics, the regulatory mechanisms guiding this process are yet to be uncovered. Here, we show that Nkx transcription factors are necessary to sustain ventricular attributes through repression of atrial identity. We recently isolated mutant alleles for *nkx2.5* and *nkx2.7,* two zebrafish homologs of *Nkx2-5*. *nkx2.5*$^{-/-}$ mutants exhibit a decrease in ventricular and an increase in atrial size. Loss of *nkx2.7* enhances this phenotype: *nkx2.5*$^{-/-}$*;nkx2.7*$^{-/-}$ mutants display a complete absence of the ventricular chamber. The initial numbers of ventricular and atrial cardiomyocytes in mutant embryos appear normal. Yet, ventricular cardiomyocytes are lost and atrial cells are gained during chamber emergence. Additionally, analysis of chamber-specific gene expression patterns highlights a switch from a ventricular marker, *vmhc*, to a marker of atrial identity, *amhc*, suggesting transdifferentiation. Furthermore, our studies indicate that the Iroquois transcription factor Irx1a acts downstream of *nkx* genes. Ventricle-specific expression of *irx1a* is downregulated in *nkx2.5*$^{-/-}$ and *nkx2.7*$^{-/-}$ mutants and is absent in *nkx2.5*$^{-/-}$*;nkx2.7*$^{-/-}$ mutants. Inhibition of *irx1a* function leads to atrial expansion and ventricular diminution, uncovering its important role in regulating cardiac chamber proportionality. Together, our results demonstrate a pivotal role for *nkx* genes in maintenance of cardiac chamber identity and underscore a previously unappreciated function of *irx1a*. These findings have the potential to uncover etiologies of congenital heart disease in patients with *NKX2-5* mutations and to direct innovations in cardiac regenerative medicine.

S7.2 Zebrafish second heart field development relies on early specification of progenitors and nkx2.5 function.

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We recently reported that latent TGFβ binding protein 3 (ltbp3) marks the zebrafish anterior second heart field (SHF) that contributes myocardium to the distal ventricle and three cardiovascular lineages to the outflow tract (OFT). However, SHF expression of ltbp3 initiates at the arterial pole of the linear heart tube, well after higher vertebrates specify SHF progenitors in the lateral plate mesoderm (LPM). Building on a recent dye tracing study from the Kirby laboratory, we identified putative nkx2.5+ SHF progenitors in the zebrafish anterior LPM (ALPM), cranial and medial to adjacent early-differentiating cardiomyocytes (nkx2.5+, cmlc2+). Using inducible Cre/loxP-mediated lineage tracing, we pulse-labeled nkx2.5+, GATA4+, or cmlc2+ cells in the ALPM and characterized their descendents using lineage specific reporters. From these analyses, we learned that pulse-labeled nkx2.5+ and GATA4+ cells give rise to myocardium in the entire ventricle and three cardiovascular li neages in the OFT. Pulse-labeling of cmlc2+ cells revealed that early differentiating cardiomyocytes reside predominantly in the proximal ventricle. Taken together, these data suggest strongly that nkx2.5+, GATA4+, cmlc2- SHF progenitors are specified in the zebrafish ALPM prior to initiating expression of ltbp3 at the arterial pole. Furthermore, we tested whether nkx2.5 plays a conserved and essential role during zebrafish SHF development. Nkx2.5 morphant embryos exhibited several SHF-related phenotypes including significant reductions in distal ventricular myocardium, outflow tract smooth muscle, and expression of ltbp3. Taken together, our data reveal two conserved features of zebrafish SHF development underscoring the utility of this model organism for deciphering SHF biology.

S7.3 Notch1 mediated signaling cascades regulate cardiomyocyte polarity and ventricular wall formation.

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Ventricular trabeculation and compaction are two anatomically overlapping morphogenetic events essential for normal myocardial development. Disregulation of these events can lead to Left Ventricular Noncompaction (LVNC, MIM300183). However, the underlying mechanisms and pathogenetic pathways remain elusive, largely due to the genetic heterogeneity of the patients and the lack of suitable animal models. Recently, through the use of gene profiling and cardiac cell-lineage restricted genetic manipulation, we analyzed a unique mouse genetic model for LVNC, *Fkbp1a* knockout mice. Our data demonstrated a critical contribution of over-activated Notch1 and subsequent neuregulin1-ErbB2/4 signaling in developing LVNC in mouse, and confirmed previous finding that Notch1-neuregulin1/Bmp10 signaling pathways in regulating ventricular cardiomyocyte proliferation. Remarkably, our new emerging data also suggested that the excessively activated Notch1-neuregulin1/ErbB2 signaling cascade dramatically down-regulated dishevelled-associated activator of morphogenesis 1 (Daam1), a member of the Formin family and a potential effector of non-canonical Wnt planer cell polarity (PCP) signaling. Genetic ablation of Daam1 resulted in disoriented cardiomyocyte polarity with altered actin cytoskeleton and myofiber organization, and concomitantly ventricular noncompaction, which confirmed the role of Daam1 in the development of LVNC. More interestingly, we have also identified that p21-activated kinase 1 (Pak1), a Ser/Thr protein kinase known to control tyrosine kinase receptor (*i.e.*, ErbB2/4) phosphorylation and small GTPases activities in regulating cell proliferation, cell polarity, and actin cytoskeleton organization, is a potential up-stream regulator of Daam1. Activated Pak1 (phospho-T423) was dramatically up-regulated in *Fkbp1a* mutant hearts with excessive activation of ErbB2/4. In addition, over-expression of constitutively active Pak1 (Pak1ca) in primary cultured neonatal cardiomyocytes down-regulated the level of Daam1. Taken together, our *in vivo* and *in vitro* data demonstrated that Notch-neuregulin-Pak1-Daam1 is an essential signaling cascade in regulating cardiomyocyte polarity and ventricular wall formation.

S7.4 Mutations in the NOTCH pathway regulator MIB1 cause ventricular non-compaction cardiomyopathy

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Ventricular non-compaction (VNC) cardiomyopathy patients show prominent trabeculations mainly in the left ventricle and reduced systolic function. Clinical presentation varies from asymptomatic to heart failure. We show that germline mutations in human MIND BOMB-1 (MIB1), encoding an E3 ubiquitin ligase that promotes endocytosis of the NOTCH ligands DELTA and JAGGED, cause VNC in autosomal-dominant pedigrees, and affected individuals show reduced NOTCH1 activity. Functional studies in cells and zebrafish embryos and in silico modeling indicate that these are loss-of-function phenotypes. VNC is triggered by targeted inactivation of Mib1 in mouse myocardium, and mimicked by inactivation of myocardial Jagged1 or endocardial Notch1. Myocardium-specific Mib1 mutants show reduced ventricular Notch1 activity, and an expansion of compact myocardium markers to the abnormally proliferative, immature trabeculae. These results identify NOTCH as a primary signaling pathway involved in VNC, and suggest that MIB1 mutations cause a developmental arrest in chamber myocardium, preventing trabecular maturation and compaction.

S7.5 The early role of *Tbx1* in anterior and posterior second heart field cells

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During cardiac development, the anterior second heart field (SHF) contributes cells to the arterial pole and the posterior SHF to the venous pole. T-box transcription factor Tbx1 is expressed in the SHF and is known to be involved in development of the arterial pole. Here, we aimed to elucidate the early role of Tbx1 in the posterior SHF and derived structures, and its relation to anterior SHF development. Using quantitative 3D reconstruction methodologies, we found that the proliferative rate and population size of SHF cells was severely affected in *Tbx1*-deficient mouse embryos. DiI labeling of anterior SHF cells in an *Fgf10* enhancer trap transgene revealed that, in the absence of *Tbx1,* the anterior movement of anterior SHF cells fails from the 7 somite stage. Moreover, these anterior SHF cells contribute to the venous pole and differentiate into atrial myocardium by activating the local atrial gene program. Distal hypoplasia of the outflow tract seems to be a late consequence of this defect. In addition, the distance between the outflow tract and the inflow tract was greatly reduced and the subsequent fusion of the dorsal mesocardium was consequently delayed. Our findings suggest that *Tbx1* is involved in maintaining the SHF population size from embryonic day (E) 8.0 onward and that *Tbx1*-deficiency disrupts proliferation, differentiation and cell fate decisions within the SHF during early heart morphogenesis. Moreover, despite prepatterning into anterior and posterior subdomains, cardiac progenitor cells of the SHF seem to be naïve in the absence of *Tbx1*.

S7.6 Retinol Dehydrogenase 10: Roles in Embryo Pharyngeal Patterning and a Model to Understand How Retinoid Gradients Prevent Congenital Birth Defects

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Retinoic acid (RA), an active vitamin A metabolite, is a key signaling molecule in vertebrate embryos. Morphogenetic RA gradients are thought to be set up by tissue-specific actions of retinaldehyde dehydrogenases (RALDHs) and catabolizing enzymes (CYP26s) and are important in preventing congenital malformations. The retinol to retinaldehyde conversion was believed to be achieved by several redundant enzymes; however, a random mutagenesis screen identified retinol dehydrogenase 10 (RDH10) as responsible for a homozygous lethal phenotype (Rdh10Trex mutants: Sandell et al., Genes Dev. 21, 1113-1124, 2007) with typical features of RA deficiency. We report the production and characterization of novel murine Rdh10 loss of function alleles generated by gene targeting. We show that, although the Rdh10-/- mutants die at an earlier stage than Rdh10Trex mutants, their molecular patterning defects do not reflect a complete state of RA deficiency. Genetic models of RA/RALDH2 deficiency p roduces hypoplastic development of the 3rd to 6th branchial arches, a malformation disrupting cardiac outflow tract septation. In Raldh2-/- mutants this defect cannot be restored by maternally administered RA. In Rdh10-/- mutants, pharyngeal growth and patterning is restored by administering retinaldehyde to pregnant mothers. We hence obtain viable Rdh10-/- mutants, free of lethal congenital malformations. This is the first demonstration of rescue of an embryonic lethal phenotype by simple maternal administration of the missing retinoid compound. These results underscore the importance of maternal retinoids in preventing congenital birth defects, and lead to a revised model on the enzymatic control of embryonic RA distribution.

Platform Session 8: Valve Development

S8.1 Computational modeling of endocardial cell activation during AV valve formation.

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The development of valve structures occurs in distinguishable phases, which are highly conserved. The first event during valve development is the induction of endocardial cushions (ECs) within the atrioventricular (AV) canal and outflow tract of the primitive heart tube. It has been well established that a BMP signal from the myocardium initiates this event by the induction of TGF-β, *Has2* (hyaluronic acid synthase 2), and Notch1, as well as the transcription factors Snail1 and Twist1. The importance of regulating *Has2* expression in the endocardium has been exemplified by the observation that in *Has2* deficient mice or zebrafish the cardiac jelly does not expand and ECs fail to form. We recently showed that zebrafish *dicer* mutant embryos, lacking mature miRNAs, form excessive endocardial cushions (Lagendijk et al. *Circ Res*. 2011). By functional screening we found that miR-23 is both necessary and sufficient for restricting the number of activated endocardial cells in the ECs. In addition, in mouse endothelial cells, miR-23 inhibited a TGF-β–induced endothelial-to-mesenchymal transition. By *in silico* screening combined with in vivo testing, we identified *Has2*, *Icat,* and *Tmem2* as novel direct targets of miR-23. Surprisingly, miR-23 expression is confined to the ECs where its activity is required to repress Has2. To understand the significance of the miR-23-Has2 interaction we applied computational modeling using a cellular Potts algorithm. The computational model predicts a regulatory network of both positive and negative feedback mechanisms to restrict the activation of the endocardial cells to the AV canal.

S8.2 Tie1 is Required for Semilunar Valve Form and Function

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The mechanisms regulating late gestational and early postnatal semilunar valve remodeling and maturation are poorly understood. Tie1 is a receptor tyrosine kinase with broad expression in embryonic endothelium. During semilunar valve development, Tie1 expression becomes restricted to the turbulent, arterial surfaces of the valves in the perinatal period. Previous studies in our laboratory have demonstrated that Tie1 can regulate cellular responses to blood flow and shear stress. We hypothesized that Tie1 signaling would regulate the flow dependent remodeling of the semilunar valves associated with the conversion from maternal/placental to independent neonatal circulation. To circumvent the embryonic lethality, we developed a floxed Tie1 allele and crossed it to an Nfatc1 P2-Cre line that mediates gene excision exclusively in the endocardial cushion endothelium. Excision of Tie1 resulted in aortic valve leaflets displaying hypertrophy with perturbed matrix deposition and re modeling without alteration in cell number, proliferation, or apoptosis. Differences were only detected after birth, increased with age, and were restricted to the aortic valve. RNA sequence analysis suggests that mutant aortic valves are similar to wildtype pulmonary valves in transcriptional profile. The aortic valves demonstrated insufficiency and stenosis by ultrasound and atomic force microcopy documented decreased stiffness in the mutant aortic valve consistent with an increased glycosaminoglycan to collagen ratio. These data suggest that active endocardial to mesenchymal signaling, at least partially mediated by Tie1, is uniquely required for normal remodeling of the aortic but not pulmonary valve in the late gestation and post-natal animal.

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S8.3 In Vivo Reduction Of Smad2 Concomitant With Proteoglycan Accumulation Results In High Penetrance Of Bicuspid Aortic Valves

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Bicuspid aortic valve (BAV) is the most prevalent cardiovascular malformation resulting in two rather than three mature cusps and occurs in at least 1-2% of the population. However, only NOTCH-1 loss of function has been linked to human BAV disease, and there are limited genetically modified mouse models that display BAV. We reported that loss of proteolytic cleavage of versican, a proteoglycan abundant in cardiac outlet cushions, results in myxomatous valves in ADAMTS5 deficient mice. Recently we discovered that a failure to cleave versican was concomitant with a reduction of cell-cell condensation, phosphorylated Smad2 and fibrous ECM organization in *Adamts5*$^{-/-}$ valves. To test the hypothesis that that versican cleavage via ADAMTS5 is required to reduce the versican-hyaluronan pericellular matrix to elicit Smad2 phosphorylation we further reduced Smad2 in *Adamts5*$^{-/-}$ mice through intergenetic cross. The resulting *Adamts5*$^{-/-}$*;Smad2*$^{+/-}$ mice developed a more dramatic valve phenotype than *Adamts5*$^{-/-;}$*Smad2*$^{+/+}$ mice and displayed a high penetrance of BAV (6/9). Interestingly, the pulmonary valve was also bicuspid in 5/9 (4/9 displayed both bicuspid PV and BAV). All *Adamts5*$^{-/-}$*;Smad2*$^{+/-}$ semilunar valves displayed wide hinge regions at the juncture of the annulus concomitant with loss of fibrous ECM. These data suggest that the dramatic changes in proteoglycan turnover, during remodeling of the truncal cushions, elicit changes in cell behavior and signaling required for normal semilunar cusp formation. Further studies of the *Adamts5*$^{-/-}$*;Smad2*$^{+/-}$ mice may elucidate a novel etiology of BAV pathogenesis and lead to new pharmacological treatments for valve disease.

S8.4 Filamin-A Regulates Tissue Remodeling of Developing Cardiac Valves via a Novel Serotonin Pathway

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Linkage and sequencing studies of patients with myxomatous valvular dystrophy revealed causal mutations in the Filamin-A gene. These studies defined Filamin-A as integral to valve structure and function. However, the mechanisms by which Filamin-A regulates valve cell biology are unknown. Herein we demonstrate that Filamin-A conditional KO (cKO) mice exhibit enlarged leaflets commencing during fetal valve maturation. This defect is caused by an inability for valve fibroblasts to efficiently remodel their extracellular matrix environment. Using the patient mutations as a guide, we identified a novel mechanism by which cooperative interaction between intracellular serotonin, transglutaminase-2 (TG2), and Filamin-A are required for promoting cytoskeletal-driven matrix remodeling events. Co-expression of serotonin, serotonin transporter (SERT), TG2, and Filamin-A is restricted to fetal valve development during active matrix remodeling. At this timepoint, immunoprecipitation experiments demonstrate that serotonin is covalently bound to Filamin-A *in vivo* and *in vitro* and is dependent on intact TG2 activity. Pharmacological and genetic perturbations of this interaction demonstrate serotonin transport inhibition, abrogation of TG2 activity, and/or genetic removal of Filamin-A result in loss of serotonin-Filamin-A interaction and impaired matrix remodeling. These findings illustrate a novel mechanism by which intracellular serotonin, TG2 and Filamin-A cooperatively regulate cytoskeletal-driven matrix remodeling. As such, these data provide mechanistic insight into normal processes driving cardiac valve maturation with added potential for providing insight into the developmental basis for human degenerative cardiac valve disease.

S8.5 Epicardially-derived Fibroblasts Preferentially Contribute to the Parietal Leaflets of the Atrioventricular Valves in the Murine Heart

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The importance of the epicardium for valvuloseptal development has been well established. To determine whether and if so how epicardially derived cells contribute to the developing valves in the murine heart we used a mWt1/IRES/GFP-Cre mouse to trace the fate of EPDCs from embryonic day (ED)10 until 4 months of age. Migration of EPDCs into the atrioventricular cushion mesenchyme starts around ED12. As development progresses, the number of EPDCs in the cushions increases significantly, specifically in the leaflets which derive from the lateral atrioventricular cushions. In these leaflets, the epicardially-derived fibroblasts eventually largely replace the endocardially-derived cells. Importantly, the contribution of EPDCs to the leaflets derived from the major AV cushions is very limited. The differential contribution of EPDCs to the respective leaflets of the atrioventricular valves provides a new paradigm in valve development and could lead to new insights into the pathogenesis of abnormalities that preferentially affect individual components of this region of the heart. The notion that there is a significant difference in the contribution of epicardially and endocardially derived cells to the individual leaflets of the atrioventricular valves has also important pragmatic consequences for the use of endocardial and epicardial cre-mouse models in heart development.

S8.6 Endothelial nitric oxide signaling regulates Notch1 in aortic valve development and disease

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Aortic valve calcification is the third leading cause of heart disease in adults and is frequently associated with bicuspid aortic valve, the most common type of cardiac malformation. NOTCH1 and the nitric oxide (NO) signaling pathway have been implicated in aortic valve development and calcification. Using an established porcine aortic valve interstitial cell (PAVIC) culture system known to spontaneously calcify, we demonstrate that gain or loss of NO, secreted by endothelial cells, prevents or accelerates calcification of PAVICs, respectively. Overexpression of Notch1 prevented the calcification that occurred with inhibition of NO, demonstrating the effects of NO on calcification are mediated by Notch1. Furthermore, endothelial cells or addition of NO regulates the nuclear localization of Notch1 and affects the expression of Hey1, a Notch1 target gene in PAVICs. To determine if Notch1 and endothelial nitric oxide (eNOS) genetically interact *in vivo*, we generated *eNOS*$^{-/-}$*;Notch1*$^{+/-}$ compound mutant mice. Embryonic examination revealed cardiac abnormalities in a subset of *eNOS*$^{-/-}$*;Notch1*$^{+/-}$ mice, which suffered ~70% lethality by postnatal day 10. Surviving compound mutant mice at 8 weeks of age demonstrated severely malformed aortic valves. *eNOS*$^{-/-}$*;Notch1*$^{+/-}$ mice maintained on high fat diet for 12 weeks displayed aortic regurgitation and stenosis, thickened aortic cusps and development of calcific nodules on the aortic valve leaflets as compared to age-matched *eNOS*$^{-/-}$ mice. Overall, these data demonstrate a novel molecular pathway by which endothelial nitric oxide regulates Notch1 signaling in a manner that is critical for aortic valve development and disease.

Moderated Poster Discussions

Session 1: Cardiac Regeneration

2.7 Cardiomyocyte proliferation contributes to post-natal heart growth in humans

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Cardiomyocyte proliferation is an active cellular mechanism during myocardial growth and regeneration in zebrafish, newt, and neonatal mice. We hypothesized that cardiomyocyte proliferation may also contribute to the growth of the human myocardium between birth and adulthood. To test this hypothesis, we analyzed the cellular growth mechanisms in a set of 20 human hearts (age 3 weeks – 20 years) that were procured for transplantation and free from myocardial diseases. Measurements of mitosis, made by automated quantification of phosphorylated histone-3-positive cardiomyocytes, showed that during the first year of life the mean percentage of cardiomyocytes undergoing mitosis was 0.05%, declining to 0.01% at 20 years of age. Cardiomyocyte cytokinesis, visualized by an antibody against MKLP-1 (a component of the centralspindlin complex), was detectable up to 20 years of age, but not later in life. The number of cardiomyocytes, quantified with stereology, increased four-fold between birth and 20 years. The mean cardiomyocyte volume increased concomitantly 12-fold. Relating these mechanistic data to left ventricular mass (determined by echocardiography) showed that cardiomyocyte proliferation contributes 37% to physiologic myocardial growth between birth and 20 years. These findings suggest that altered cardiomyocyte proliferation may be involved in abnormal myocardial growth in congenital heart disease. Our findings also suggest that myocardial regeneration may be present or stimulated in young humans since the underlying mechanism, cardiomyocyte proliferation, is active under the age of 20 years.

2.8 Microarray and Ultrastructure Analyses of a Regenerative Myocardium

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Ciona intestinalis is an invertebrate animal model system that is well characterized and has many advantages for the study of cardiovascular biology. A striking difference between most vertebrates and Ciona is that the Ciona myocardium is capable of regenerating cardiac myocytes throughout its lifespan, which makes the regulatory mechanisms of cardiac myocyte proliferation in Ciona intriguing. In order to identify important regeneration factors in Ciona, microarray analysis was conducted on RNA from adult Ciona hearts with normal or damaged myocardium using custom Affymetrix GeneChips. Hearts were injured via ligation or cryoinjury to stimulate regeneration. After a 24 or 48 hour recovery period, total RNA was isolated from damaged and control hearts. Initial results indicate significant changes in gene expression in hearts damaged by ligation in comparison to cryoinjured or control hearts. Ligation injury shows differential expression of 223 genes as compared to control (fold change >2, $p<0.01$, Student's t-test) with limited false discovery (5.8%). Among these 223 genes, 117 have known orthologs of which 68 were up-regulated and 49 were down-regulated. Ultrastructure analyses of injured myocardium using TEM were conducted in parallel to the microarray study. Preliminary results show changes in the myofibril arrangement and cellular organization in injured hearts. Further studies using immunohistochemistry to identify proliferation and apoptosis in cardiac myocytes of damaged hearts are currently underway. Taken together, these studies will coordinate differences in gene expression to cellular changes in the regenerative myocardium of Ciona, which will help to elucidate the regulatory mechanisms of cardiac myocyte proliferation.

2.9 Deciphering novel molecular mechanisms that facilitate cardiomyocyte de-differentiation and proliferation

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Mammalian cardiomyocytes (CMs) withdraw from the cell cycle shortly after birth and thus lack significant renewal and regenerative capacity. Therefore, inducing CM de-differentiation to enable their cell cycle re-entry

could provide the basis for a therapeutic approach for the treatment of cardiac dysfunction following myocardial infarction. In contrast to the adult mammalian heart, the heart of one-day old mice maintains the capacity to completely regenerate after partial resection, by proliferation of mature CMs. The main objective of our work is to decipher the structural and molecular differences between CMs of adult versus neonatal hearts, in attempts to improve the regenerative capacity of the adult mammalian heart. For that, we use multidisciplinary approaches enabling to study the molecular and cellular mechanisms that govern CM proliferation, combining various structural and molecular cell-biology methodologies, using both cell lines and primary cell cultures. Specifically, we characterized CM populations derived from 1d and 7d old mice and determined the expression profile of their cardiogenic genes, as well as their proliferation, differentiation and de-differentiation capacity in vitro. In addition to this characterization, we study how the composition, geometry and forces of the extracellular environment affect the specific features of CMs. Using high-throughput live-cell imaging platforms, we test various signalling pathways for their ability to prime the proliferation, differentiation and de-differentiation of CMs. Preliminary results and future directions of the combined strategy of studying both the traits of CMs together with those of the microenvironment and the possible crosstalk between will be described.

2.10 Tissue specific translational profiling during zebrafish heart regeneration

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Zebrafish have a robust ability to replace lost cardiomyoyctes, and thus provide a unique model to dissect heart regeneration. Genetic lineage-tracing has revealed that regeneration occurs by activating proliferation of pre-existing cardiomyocytes at the injury site. In particular, cardiomyocytes that activate expression of the transcription factor Gata4 are the major contributors of new cardiac muscle. Yet, the molecular mechanisms by which injury activates cardiomyocyte proliferation remain elusive. Here, we have applied the recently developed technology, translational affinity purification (TRAP), to obtain genome-wide profiles of translated mRNAs from different cardiac cell types during regeneration. We created transgenic lines to profile cardiomyocytes, gata4-positive cardiomyocytes, epicardium, and endocardium, at various times after two different models of cardiac injury. Through translational profiling of cardiomyocytes, we have begun to identify new injury responses by cardiomyocytes that are critical for heart regeneration in zebrafish.

Session 2: Human Genetics & Disease Models

1.7 Novel role of altered cardiac function in the progression of heart defects in Fetal Alcohol Syndrome (FAS)

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Over 500,000 American women per year report drinking alcohol during pregnancy, with 1 in 5 who also binge drink. Even low levels of prenatal alcohol/ethanol exposure can produce birth defects in humans. Epidemiological studies suggest that 54% of live-born children with Fetal Alcohol Syndrome (FAS) present with cardiac anomalies. While the mechanisms of ethanol exposure have been studied extensively, most studies fail to consider the role of altered cardiac function in producing congenital heart defects. It is already known that changes in hemodynamics can profoundly affect cardiac development. We hypothesized that ethanol exposure creates early hemodynamic anomalies which contribute significantly to subsequent cardiac structural and functional defects. We employed optical coherence tomography (OCT), which is a non-destructive imaging modality capable of real-time, micrometer-scale resolution imaging. OCT allowed us to accurately map changes in hemodynamic forces (e.g. shear stress) and the resultant structural abnormalities in the live embryo at very early stages, when the trajectory to heart defects can begin. In our studies, avian embryos exposed to ethanol during gastrulation exhibited alterations in overall embryo body flexure, blood flow, shear stress and cardiac cushion development during heart looping stages. A combination of myo-inositol (MI) and folate (FA) given at gastrulation stages are known to prevent ethanol-induced cardiac abnormalities in mouse/avian models. In future studies, we will test whether FA/MI supplementation rescues these defects through normalization of cardiac function. Our contributions could be a first step in implementing new therapeutic strategies based on FA/MI prevention of birth defects. Research Support: HL083048, HL095717.

1.8 Noonan syndrome associated RAF1 mutant evokes hypertrophic cardiomyopathy features in human cardiomyocytes in vitro.

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Noonan syndrome (NS) and its related disorders, which are now called “RASopathies”, are caused by aberrant activation of RAS/ERK pathway. Most RASopathies feature proportional short stature, facial dysmorphia, cognitive impairment and cardiac defects. The cardiac manifestations in RASopathies vary widely, but hypertrophic cardiomyopathy (HCM) is found in virtually all NS cases caused by RAF1 allele that encode a hyperactive kinase mutant. HCM also is common in LEOPARD syndrome and Costello syndrome. We reported previously that a mouse model of NS caused by a kinase-activated Raf1 mutant recapitulates major features of NS, including HCM. Importantly, these features were normalized by post-natal treatment of MEK inhibitor. To extend these mouse studies to pre-clinical human models to better identify detail molecular basis and signaling as well as to aid in the development of new therapies for RASopathies, we have generated human induced pluripotent stem cells (hiPSCs) from fibroblasts of multiple RASopathy patients. We found that a kinase-activating RAF1 mutant causes increased cell size of cardiomyocytes differentiated from hiPSCs compared with normal hiPSCs. We also found increased calcium sensitivity and lower response to b adrenergic stimulation when we cultured differentiated cardiomyocytes as engineered heart tissue, which allows us to apply mechanical forces on these cardiomyocytes. Our data show that NS associated RAF1 mutant can cause HCM phenotypes in vitro and provide a potential pre-clinical system for testing new therapies.

1.9 Engineering new mouse models to map dosage-sensitive genes in Down syndrome congenital heart defects

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Trisomy of human chromosome 21 (Hsa21) occurs in ~1 in 750 live births and the resulting gene dosage imbalance causes Down syndrome (DS). One of the most important medical aspects are the congenital heart defects (CHD), which affect around half of all individuals with DS. The defects observed in DS range from simple chamber septation to complex CHD involving multiple aspects of the heart anatomy. The most common CHD in DS affect the atrio-ventricular (AV) junction. Using high-resolution episcopic microscopy (HREM) in mouse embryonic hearts, we have studied a ‘transchromosomic’ (Tc1) mouse model of DS, which carries Hsa21 as a freely segregating chromosome. The Tc1 mouse closely resembles human DS CHD, replicating many of the features of the AV septal defects found in DS (Dunlevy, 2010). To unravel the mechanisms of defective heart formation in the Tc1 mouse embryo, we are using lineage markers to study the tissues involved in development of the AV junction. In order to identify dosage-sensitive genes that may underlie DS CHD and other complex biological effects of trisomy, we are generating a high-resolution mapping panel of mouse strains with partial trisomies and monosomies for regions of mouse chromosomes orthologous to Hsa21. Using systematic HREM analysis, we are examining partial trisomic strains to identify regions that are sufficient to induce the CHD phenotype. Conversely, by crossing Tc1 mice to partial monosomic strains and assaying the effect of each interval on the cardiac phenotype, we are testing individual genes for their potential role in DS CHD.

1.10 Recovery of ENU induced mutations causing congenital heart disease using next-gen sequencing

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Forward genetic screens provide a powerful non-gene biased approach to elucidate the genetic basis for congenital heart disease (CHD). In the past this has been hampered by difficulties in mutation recovery. In our current large-scale recessive mouse ENU mutagenesis screen, we are pursuing the use of whole genome/whole exome sequencing for mutation recovery. To evaluate the efficacy of our mutation recovery pipeline, the mutant Destro exhibiting complex CHD with heterotaxy was analyzed by both whole exome and whole genome sequencing. A multi-step bioinformatics filtering strategy was developed for mutation recovery. First, we removed sequence variants present in dbSNP129 or our in-house mouse databases. Second, coding

variants likely to be homozygous based on sequence coverage, unique reads, and strandedness were prioritized as potential candidates. Third, mutations are further rank ordered based on cross-species conservation. Finally, the causal mutation is identified by genotyping multiple mutants with the same phenotype. Whole-genome sequencing of Destro with 8x coverage together with our filtering strategy reduced 178,848 variants to just 6, with genotyping analysis identifying Bicc1$^{c.606+2T>C}$ as the disease-causing mutation. This same mutation was also recovered by whole-exome sequencing with ~50x target-coverage. In another mutant line 370 with coarctation and diaphragmatic hernia, exome sequencing analysis yielded a single mutation in Lox1, which was confirmed to be the disease causing mutation. Over 10 mutations have been recovered thus far. These results suggest our whole exome-sequencing pipeline will provide a cost-effective strategy for meeting our goal of pursuing a saturation level mutagenesis screen to recover mutations causing CHD. Supported by funding from U01-HL098180

Session 3: Transcriptional Regulation

3.7 Mutual Regulation of Nkx2.5 and Fibulin-1 in the SHF Regulatory Network

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Approximately 12,000 children are born yearly with malformations of the aorta and/or pulmonary artery resulting from abnormal outflow tract (OFT) development. The cardiac OFT arise from progenitor cells of the second heart field (SHF) and animal studies have shown that this process is subject to the influence of several transcription factor, growth factor and extracellular matrix (ECM) genes. It is therefore likely that the combinatorial effects of multiple gene mutations underlie genetically complex OFT congenital heart defects (CHD) entities in humans. As part of an effort to determine functional linkages between known OFT developmental regulators, we have found evidence for mutual regulation of the cardiac transcription factor Nkx2.5 and the ECM protein fibulin-1 (Fbln1), both of which are required for normal SHF and OFT development in mice. Specifically we have found that Fbln1 expression levels are decreased in SHF regions in Nkx2.5 null mice, and that Nkx2.5 appears to regulate Fbln1 expression in the SHF via binding to an evolutionarily conserved NKE binding site in promoter proximal regions. Conversely, Fbln1 expression reciprocally regulates Nkx2.5, as Nkx2.5 expression is decreased in SHF regions of Fbln1 null mice in association with increased expression of a potential direct repressor of Nkx2.5, the growth factor activated transcription factor Egr1. These findings support the hypothesis that Nkx2.5 and Fbln1 are key components of a growth factor-mediated homeostatic mechanism regulating SHF proliferation and OFT development.

3.8 FOG-2 Mediated Recruitment of the NuRD Complex Regulates Cardiomyocyte Proliferation during Heart Development

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FOG-2 is a multi-zinc finger protein that binds GATA4 and modulates GATA4-mediated transcriptional control of target genes during heart development. Our previous work has demonstrated that the Nucleosome Remodeling and Deacetylase (NuRD) complex physically interacts with FOG-2 and is necessary for FOG-2 mediated repression of GATA4 activity in vitro. In this report, we describe the generation and characterization of mice homozygous for a mutation that disrupts FOG-2/NuRD complex interaction (FOG-2^{R3K5A}). These mice exhibit a perinatal lethality and have multiple cardiac malformations, including a ventricular septal defect and a thin ventricular myocardium. To investigate the mechanism underlying the thin ventricular walls, we measured the rate of cardiomyocyte apoptosis and proliferation in wild-type and FOG-2^{R3K5A} developing hearts. We found no significant difference in the rate of cardiomyocyte apoptosis between wild-type and FOG-2^{R3K5A} mice. However, cardiomyocyte proliferation was reduced by 29.8% in FOG-2^{R3K5A} mice. Interestingly, we found by gene expression analysis that the cell cycle inhibitor p21 is up-regulated 1.7-fold in FOG-2^{R3K5A} hearts. Further, we demonstrate that FOG-2 can directly repress the activity of the p21 gene promoter using an in vitro transient transfection assay. In addition, chromatin immunoprecipitation (ChIP) reveals that the NuRD complex is bound to the p21 promoter in wild-type embryonic day 14.5 hearts but is diminished at this promoter in mutant hearts. Taken together, our results suggest the FOG-2/NuRD interaction is required for cardiomyocyte proliferation by specifically down-regulating expression of the cell cycle inhibitor p21 during heart development.

3.9 A strict lineage boundary between the first and second heart fields is defined by the contribution of the Tbx5 lineage.

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The theory that the heart arises from two distinct heart fields suggests important clues as to how the heart is patterned. Determining if contributions of distinct first heart field (FHF) and second heart field (SHF) cell populations to the fully formed heart exist is central to understanding heart development and the etiology of congenital heart defects (CHDs). Haploinsufficiency of the transcription factor TBX5 causes Holt-Oram Syndrome, which includes CHDs. Tbx5 expression outlines a region of the heart that is presumed to correspond to the FHF, and Tbx5 is required in the posterior half of the heart. However, it is not known what structures within the heart the Tbx5 lineage gives rise to, or how these relate to the importance of Tbx5 in CHDs. Using an inducible Cre recombinase inserted at the Tbx5 locus, and various SHF genetic markers, we have mapped the Tbx5-expressing lineage and its intersection with the SHF, to discern field allocation in cardiac morphogenesis. We fi nd that from the earliest stages of Tbx5 expression, prior to formation of the linear heart tube, the Tbx5 lineage contributes to the posterior segments of the heart, ending at a sharp boundary at the interventricular septum, with little overlap with the SHF. We propose that the earliest Tbx5 expressing cells define the FHF lineage, and that a strict lineage boundary exists between the FHF and SHF prior to morphogenic distinctions between their descendents. Understanding the contribution of Tbx5 to the FHF will contribute to our knowledge of heart development and CHDs.

3.10 Tbx5-Hedgehog pathway is required in second heart field cardiac progenitors for atrial septation

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The developmental mechanisms underlying human Congenital Heart Disease (CHD) are poorly established. Atrial Septal Defects (ASDs), a common form of CHD, can result from haploinsufficiency of cardiogenic transcription factors including Tbx5. We demonstrate that Tbx5 is required outside the heart in second heart field (SHF) cardiac progenitors for atrial septation in mice. Conditional Tbx5 haploinsufficiency in the SHF, but not in the myocardium or endocardium, caused ASDs. Tbx5 SHF knockout embryos lacked atrial septum progenitors. We identified SHF Tbx5- responsive cell cycle progression genes, including cdk6 as a direct target, and Tbx5 mutant SHF progenitors demonstrated a mitotic defect. Genetic and molecular evidence including the rescue of atrial septation in Tbx5 mutant embryos by constitutive Hh-signaling placed Tbx5 upstream or parallel to Hh signaling in cardiac progenitors. We identified Gas1, a Hh-pathway member upstream of Smo, as a direct target of Tbx5. We also found that Osr1, required for atrial septaion in mice, is expressed in a Tbx5-dependent manner. We further identified Osr1 as a direct target of Tbx5 required for atrial septation. These results describe a SHF Tbx5-Hh-signaling network and independent Tbx5-Osr1 pathway required for atrial septation. Ongoing work is aimed at identifying molecular links between these pathways in atrial septation. A paradigm defining molecular requirements downstream of Tbx5 in cardiac progenitors as organizers of cardiac septum morphogenesis has implications for the ontogeny of CHD.

Session 4: Coronary Artery Development

5.7 The BMP regulator BMPER is necessary for normal coronary artery formation

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Connection of the coronary vasculature to the aorta is one of the last essential steps of cardiac development. However, little is known about the signaling events that promote normal coronary artery formation. The bone morphogenetic protein (BMP) signaling pathway regulates multiple aspects of endothelial cell biology but has not been specifically implicated in coronary vascular development. BMP signaling is tightly regulated by numerous factors, including BMP-binding endothelial cell precursor-derived regulator (BMPER), which can both

promote and repress BMP signaling activity. Analysis of the BMPER-/- mouse indicated that this deficiency leads to embryonic death, and in the embryonic heart, BMPER expression is limited to the endothelial cells and the endothelial-derived cushions, suggesting that coronary vascular defects may be present. Histological analysis of BMPER-/- embryos at embryonic day 16.5 revealed that the coronary arteries were either atretic or connected distal to the semilunar valves. However, analysis of earlier embryonic stages showed that the coronary plexus began differentiating normally and that apoptosis and cell proliferation were unaffected in BMPER-/- embryos. In vitro tubulogenesis assays showed that isolated BMPER-/- endothelial cells had impaired tube formation compared to wild-type endothelial cells. Together, these results indicate that BMPER-regulated BMP signaling is critical for the migration of coronary endothelial cells and normal coronary artery development.

5.8 Epicardial chemokine signaling influences ventricular wall proliferation and coronary vasculogenesis

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During heart development, the epicardium secretes soluble factors that direct the morphological organization of the underlying myocardial wall. Our previous study established that insulin-like growth factor 2 (IGF2) is the primary mitogen expressed by the epicardium that controls embryonic ventricular cardiomyocyte proliferation. We found that CXCL12 (stromal derived factor-1) is an additional factor expressed by epicardial cells in vitro and by the epicardium in vivo. Cultured primary cardiomyocytes treated with epicardial conditioned media or in coculture with epicardial cells show a proliferative response that is partially blocked by AG1024 (an IGFR inhibitor) or AMD3100 (a CXCR4 inhibitor), and almost completely abolished by both compounds together. To date, a functional role for CXCL12 signaling in heart development has not been examined. Our results indicate that both global Cxcl12 null and cardiac-specific (Nkx2.5Cre) Cxcr4 null embryos show a moderate deficiency in compact zone proliferation. Embryos with combined loss of IGF2 and CXCL12 signaling exhibit pronounced edema, suggesting that epicardial IGF2 and CXCL12 together promote mitogenic signaling needed for cardiomyocyte proliferation and compact zone expansion. Because CXCL12 is a chemokine that has known effects on vascular cells, we also examined coronary vessel formation in embryos with disrupted CXCL12 signaling. We observed expanded superficial vessels and a reduced number of intramyocardial arteries. Our results indicate that the epicardium is a source of secreted CXCL12 that participates with the major mitogen IGF2 to induce cardiomyocyte proliferation and compact zone expansion, and also acts on endothelial cells to support formation of the coronary vasculature.

5.9 The role of Pod1/Tcf21 in epicardium-derived cells during cardiac development and fibrosis

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During cardiac development, epicardium-derived cells (EPDCs) differentiate into fibroblasts and vascular smooth muscle (SM) cells. In the adult heart, EPDCs are reactivated upon cardiac injury. Immunofluorescence studies demonstrate that the transcription factors (TF) Pod1/Tcf21, WT1, Tbx18, and NFATC1 are expressed heterogeneously in EPDCs in chicken and mouse embryonic hearts. Expression of Pod1 and WT1, but not Tbx18 or NFATC1, is activated with all-trans-retinoic acid (RA) treatment of isolated avian EPDCs. In intact E7 chicken hearts, RA signaling is required for full Pod1 expression and RA treatment inhibits SM differentiation. The requirements for Pod1 in differentiation of EPDCs in the developing heart were examined in mice lacking Pod1. Loss of Pod1 in mice leads to epicardial blistering, increased SM differentiation on the surface of the heart, and a paucity of interstitial fibroblasts, with neonatal lethality. On the surface of the myocardium, expression of multiple SM markers is increased in Pod1-deficient EPDCs, demonstrating premature SM differentiation. Together, these data demonstrate a critical role for Pod1 in controlling EPDC differentiation into SM during cardiac development. In adult hearts, cardiac injury leads to reactivation of fetal genes including WT1, Tbx18, and RALDH2 in EPDCs. Initial studies demonstrate that Pod1/Tcf21 expression is increased in mouse hearts subjected to myocardial infarction, transverse aortic constriction, or chronic isoproterenol infusion. Additional data will be presented describing Pod1/Tcf21 expression in mouse models of cardiac fibrosis. Together this work provides evidence for conserved Pod1/Tcf21-related regulatory mechanisms in EPDCs during cardiac development and fibrotic disease.

5.10 Epicardial GATA factors regulate coronary endothelial migration via Sonic hedgehog signaling.

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Early coronary vasculogenesis is dependent on the epicardium as a source of progenitor cells and regulatory signals. GATA transcription factors have been shown to be required for the formation of the proepicardium, a group of cells that give rise to the epicardium This suggests GATA factors may play a role in epicardial function. To address whether epicardial GATA-4 and GATA-6 may have a role in early coronary vascular development, we utilized a Wilm's Tumor-1 promoter-driven Cre recombinase construct to conditionally knockout GATA-4 and GATA-6 in the epicardium. We found the combined loss of GATA-4 and GATA-6 resulted in embryonic lethality at E15.5, an age at which various coronary vascular defects commonly lead to lethality. Immunofluorescent imaging at E13.5 and E14.5 indicated an 80-90% decrease in sub-epicardial endothelial cells, which are required for coronary plexus formation. This led us to hypothesize that the decreased number of sub-epicardial endothelial cells is due to a loss of epicardial signaling to early endothelial or progenitor cells. Sonic hedgehog is an epicardial signaling factor required for the migration of sub-epicardial endothelial cells. Therefore, we immunofluorescently stained for Sonic hedgehog and found its expression was lost in the sub-epicardial space of the conditional knockout. These results are consistent with a novel role for epicardial GATA factors in coronary vascular development through regulation of a Sonic hedgehog signaling pathway

Session 5: Valve Calcification & Development

8.7 Analysis of TGFβ3 function in valve remodeling and aortic valve calcification

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Transforming Growth Factor beta (TGFβ) ligands function during the onset and progression of aortic valve calcification remains obscure as both contradictory elevated and suppressed TGFβ signaling has been found in patients of calcific aortic valve disease (CAVD). Here, using in vitro micromass cultures of mouse valve precursor cells (tsA58-AVM) we showed by complimentary realtime QPCR and synthetic luciferase reporter assays that TGFβ3 can upregulate Sry-related high-mobility-group box 4 (SOX4) transcription. Sox4 is initially expressed within the endocardially-derived tissue of both the outflow tract and atrioventricular canal during development, but persists within adult valve mesenchyme. Moreover, TGFβ3-dependent regulation of Sox4 can be blocked by a small molecule inhibitor of SMAD3 (SIS3), indicating that SMAD3 acts as a downstream mediator of TGFβ3-dependent Sox4 induction. Significantly, in situ hybridization revealed that TGFβ3 is the most prevalent TGFβ isoform in postnatal heart valves. Histology demonstrated that both$Tgfb3^{-/-}$ fetal (E14.5) and $Smad3^{-/-}$ adult (6 month old) valves were abnormally thickened. There was no calcification in the aortic valves of $Smad3^{-/-}$mice. Correlative analysis of human valve tissues via immunohistochemistry (5 controls, 8 CAVD, 4 non-calcific aortic valve disease) demonstrated diffused expression of SOX4 in the control tissues, which was extinguished in the diseased tissue specimens. Interestingly, the expression of SOX4 around the calcific nodules remains upregulated within the diseased aortic valves from CAVD patients. Consistently, Sox4 siRNA knockdown in tsA58-AVM cells significantly decreased calcification. Overall, our results indicate that TGFβ3-dependent and SMAD3-mediated regulation of SOX4 may play an important role in valve remodeling and aortic valve calcification. Since small molecule inhibitor/s of SMAD3 could be used as a therapeutic approach, these data provide novel basic science rationale with potential to translate into different therapies for patients of CAVD.

8.8 Nuclear exclusion of Sox9 in valve interstitial cells is associated with calcific phenotypes in heart valves

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Calcific aortic valve disease (CAVD) is a major public health problem with no effective treatment available other than valve replacement surgery. Despite the clinical significance, the mechanisms of pathogenesis are poorly understood. Normal valve structures are composed of organized layers of extracellular matrix interspersed with valve interstitial cells (VICs) that share molecular phenotypes with cartilage, including Col2a1. In contrast, calcified valves are characterized by mineralized matrix and increased expression of osteogenic genes including Spp1. We have shown that Sox9 is required in vivo to promote the cartilaginous matrix and prevent calcification of healthy valves by directly activating Col2a1 and suppressing Spp1 in the nucleus. In this study, we show that in aortic valves from non-diseased subjects, Sox9 expression is restricted to the nuclei of VICs, while nuclear expression is downregulated and notably cytoplasmic in tissue collected from CAVD patients. Usin g a pharmacological approach, we show that Sox9-mediated calcification as a result of nuclear exclusion can be induced by retinoic acid (RA) treatment in vivo and in vitro. This decrease in nuclear Sox9 localization is associated with decreased Col2a1 expression and transactivation, activation of the Gene Ontology 'bone development' program and matrix mineralization. Ongoing studies include defining the signaling pathways that maintain Sox9 nuclear localization to prevent CAVD in healthy valves. Together, these data suggest that Sox9 nucleocytoplasmic shuttling of Sox9 plays a role in valve pathogenesis and provides insights to aid in the development of new therapeutic strategies for CAVD.

8.9 Runx2 isoforms have divergent roles in development and pathology in the heart

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Formation of atrioventricular (AV) valves in the heart begins with an epithelial-mesenchymal cell transition (EMT). In the exploration of EMT, we investigated the presence of the transcription factor, Runx2, in the AV canal. Immunostaining and PCR showed that expression coincided with the onset of EMT. siRNA treatment of AV canal cultures established that it was required for EMT and that loss specifically prevented the early cell separation step of EMT. Sequence analysis showed that the Runx2 isoform present was Runx2-I and that this isoform was regulated buy TGFβ2 but not BMP. Inhibition of Runx2-I with siRNA produced a loss of mesenchymal cell markers. Comparison with siRNA towards Snail2, suggests that the two EMT transcription factors diverge in their regulation of downstream components. To determine whether the activity of Runx2-I was conserved in other EMT events, we examined human tissue samples in the progression of esophageal adenocarcinoma. Runx2-I was e xpressed coincident with the transition from columnar epithelia (Barrett's Esophagus) to dysplasia, suggesting a conserved role in early EMT. The Runx2-II isoform is associated with bone and calcifying tissues. To compare isoform activities, we examined Runx2 isoform expression in whole valve cultures from chicks and in micromass cultures of mouse valvular mesenchymal cells. Runx2-II inceases in culture consistent with expression of calcification markers including osteopontin and matrix gla protein and with positive staining for calcium. Together, the data show a requirement for TGFβ-regulated Runx2-I in EMT and BMP-regulated Runx2-II with calcification and calcified tissues. Supported by NHLBI HL82851 and Edwards Life Sciences.

8.10 3D cardiac valve tissue reconstruction and quantitative evaluations of tissue & cell phenotypes in the Pdlim7 knock-out mouse.

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The actin binding protein Pdlim7 is part of a family of proteins involved in regulating actin dynamics. Pdlim7 is expressed throughout cardiac development and into adulthood, and inactivation in zebrafish and mouse models results in cardiac valve malformations. A recent model for atrio-ventricular (AV) valve formation and maturation describes the differential contribution of epicardially- and endocardially-derived cells to specific leaflets of the mouse valvuloseptal complex. Using the Pdlim7 knock-out mouse, we investigated whether the aberrant morphology of the adult mitral valve is caused by the distinct lineages that shape the lateral and septal leaflets. We integrate 3D heart valve reconstructions with novel methods to quantitatively test morphological differences between wildtypes and mutants. The quantitative analysis of valve shape demonstrates that our method can identify subtle, statistically significant morphological changes following loss of Pdlim7. From Pdlim7's role in actin dynamics, it is possible that the valve tissue difference is the result of aberrant cellular migration and/or cellular shape. To investigate this, we present a 3D method developed to measure cell migration behavior, and introduce the field of 3D geometric morphometrics to studies of morphogenesis and statistically evaluate the

patterns of cell shape diversity. This work in mice suggests Pdlim7 functions in AV valve remodeling and maturation at late embryonic and postnatal stages corresponding with the arrival of cells from the epicardial lineage. These findings are important because they describe a mouse model that links a novel gene (Pdlim7) to post-natal valve maturation and growth.

Session 6: Stem Cells

6.7 Dynamic Mesp1 regulation directs cardiac myocyte formation in embryonic stem cells

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Mesp1 sits on the top of the hierarchy of cardiac gene regulation network. It emerges at the onset of mesoderm formation and disappears at the cardiac crescent. The extracellular cue that triggers Mesp1, and how the Mesp1-expressing cells differentiate into cardiovascular cell lineages are largely unknown. In this study, we have crossed the $Mesp1^{Cre/+}$ and $Rosa^{EYFP/EYFP}$ mouse strains, and established an ES cell line with the genotype $Mesp1^{Cre/+}/Rosa^{EYFP/+.}$ By isolating EYFP (+) cells on day 4 of an ES differentiation protocol, we characterized the early cardiac progenitors. These cardiac progenitors enriched cardiac mesoderm markers (Flk1, PDGFRa, dHand), cardiac transcription factors (Nkx2-5, Tbx5, Mef2c), and excluded pluripotent markers (Oct4, Sox2, Nanog) and nascent mesoderm markers (T, Fgf8). Among a group of growth factors, BMP2/4 greatly induced the prevalence of EYFP (+) cells, while Wnt3a and Activin had only marginal effects. EYFP(+) cells represented a sub-population of the Flk1(+)/PDGFRa(+) cells, which were previously demonstrated to differentiate into cardiac lineages. Surprisingly, short period of BMP4 treatment (day 0-2) led to the induction of EYFP(+) cells and subsequent cardiac myocyte formation, whereas extended BMP4 (day 0-4) led to even more EYFP (+) cells but without subsequent cardiac myocyte formation. By introducing a pCAGG-loxP-bgeo-polyA-loxP-Mesp1 cassette into this ES cell line, we achieved extended Mesp1 expression in EYFP(+) cells beyond the point that endogenous Mesp1 disappears, and proved that extended Mesp1 expression impaired cardiac myocyte formation. In summary, we have established an ES cell line that allows us to trace the fate of Mesp1-expressing cardiac progenitors. Using this line as the tool, we demonstrate that dynamic Mesp1 regulation directs cardiac myocyte formation in ES cells.

6.8 Human amniocytes contain subpopulations of stem cells that have a repressed cardiogenic status.

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Amniocytes are an intriguing candidate source of stem cells for autologous repair of congenital heart defects (CHD) in humans. Despite mesodermal fate poising, amniocytes are resistant to differentiating into fully functional cardiomyocytes. Here, we provide evidence that cultured amniocytes are a heterogeneous subpopulation of stem cells expressing key pluripotency and self-renewal markers. Interestingly, qPCR analysis of 17 independent amniocyte isolates also detected the expression of the core cardiogenic factors Mef2c, Tbx5, and Nkx2-5, as well as the cardiac chromatin regulator Baf60c (Smarcd3). Gata4 is not expressed, but some individual isolates show signs of a low level of gene leakiness. However, Tbx5 and the early-to-mid cardiac markers Mesp1, Isl1, and Gata6 were consistently detectable by both qPCR and immunostaining. Stimulating amniocytes with signals known to promote differentiation of cardiac lineages failed to upregulate mRNA transcript or protein levels of core cardiogenic factors or generate cells that stain positive for cardiac troponin T (CTNT) and MF20 markers. To more precisely define the cardiogenic status of amniocytes, we performed next generation RNA-seq analysis on amniocytes. Genome-wide expression profiling reveals a paradoxical molecular signature of cardiac markers, indicating that the developmental status of amniocytes as true cardiac progenitors is incomplete, yet uniquely poised. Amniocytes strongly express multiple ES cell and mesodermal repressors that may prevent cells from progressing further down a cardiogenic lineage. These results indicate that despite an incomplete cardiogenic phenotype, direct transdifferentiation and gene knockdown approaches may allow us to overcome these barriers and drive them to a mature cardiac cell fate.

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6.9 The Homeobox Transcription Factor, Irx4, Identifies a Mutipotent, Ventricular-specific Cardiac Progenitor

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Cardiac progenitors have been presented as potential cell therapeutics, due to their cardiogenic potency. Iroquois homeobox protein 4 (Irx4) is the earliest known marker of ventricular myocardium differentiation, and the transcription factor is restricted to the ventricles throughout embryogenesis and into adulthood. Our goal is to identify a multipotent, ventricular-specific cardiac progenitor. We have targeted the 3' end of the Irx4 locus using a recombineering approach to insert fluorescence (mCherry), bioluminescence (luciferase), and antibiotic resistance (hph) cassettes. Six mESC clones were properly targeted $Irx4^{luc\text{-}mCh\text{-}hph/wt}$ ES cells. RT-PCR, western blot analysis, and immunofluorescence assays demonstrated the functional integration of the reporters during embryoid body (EB) differentiation. Following 4 days of differentiation of the $Irx4^{luc\text{-}mCh\text{-}hph/wt}$ cells in EBs, selection with hygromycin was carried out for 2 days, and day 6 cells were plated onto STO cell feeder layers for expansion. Selected $Irx4^{+}$ cells are proliferative, expressing the cell cycle antigen, Ki67, and could be passaged more than 12 times. The $Irx4^{+}$ cells express cKit, Flk1, and CXCR4 on the cell surface. Selected $Irx4^{+}$ cells demonstrate cardiovascular potency when re-aggregated and differentiated in hanging drops resulted in ventricular myocyte-enriched cell preparations (65±3.7% $cTnT^{+}$; 67±2.6% $Myl2^{+}$, 22±3.1% $SmMHC^{+}$, and 6±2% $CD31^{+}$ cells. The selected $Irx4^{+}$ population represents the first ventricular-specific multipotent progenitor population identified and holds promise for generating all cardiovascular lineages necessary to reconstitute infarcted myocardium.

6.10 Modeling the vascular phenotype of Williams-Beuren syndrome using induced pluripotent stem cells

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Elastin is essential for arterial morphogenesis. Elastin haploinsufficiency in Williams-Beuren syndrome (WBS) leads to increased vascular smooth muscle cell (SMC) proliferation and vascular stenoses. We investigated the role of elastin in proliferation and differentiation of SMCs derived from human embryonic stem cells (hESCs), and WBS patient-derived induced pluripotent stem cells (hiPSCs). Human iPSCs were reprogrammed from skin fibroblasts from a WBS patient and BJ healthy control using 4-factor retrovirus reprogramming. hESCs and hiPSCs underwent directed differentiation to generate smooth muscle cells (SMCs). Differentiated cells were treated with a peptide or an anti-proliferative drug for 6 days. mRNA and protein expression of elastin, smooth muscle α-actin (SMA), Ki67 (proliferation marker) was assessed by high-content imaging, qRT-PCR, and flow cytometry. Vascular tube formation was assessed by 3D matrigel assays. Treatment of hESC-derived SMCs increased elastin expression, increased SMA+ cells, and reduced SMC proliferation. The reprogrammed fibroblasts expressed pluripotency genes and differentiated into all germ layers. SMC differentiation was confirmed by SMA, SM22a, myocardin, smoothelin expression and by contractile response to carbachol. When compared to BJ SMCs, WBS SMCs showed lower expression of elastin protein and mRNA which increased with treatment. WBS cells showed impaired SMC differentiation, increased SMC proliferation, and impaired vascular tube formation compared to BJ cells. Treatment with an elastin ligand and a candidate drug reduced SMC proliferation, enhanced SMC differentiation, and enhanced tube formation. In conclusion, WBS iPSCs demonstrated increased SMC proliferation and impaired SMC differentiation which was partially ameliorated with treatment. Our study provides an *in vitro* platform for modeling vascular disorders and future drug screens.

General Poster Sessions

Section 1: Human Genetics & CHD Models

S1.1 Deletion of the extracellular matrix protein *Adamtsl2* results in a inter-ventricular septal defect in mice

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Geleophysic dysplasia (GD) is a rare human genetic disorder presenting with short stature, joint contractures and thick skin. High morbidity, and frequently, juvenile mortality results from cardiac valvular and tracheo-pulmonary anomalies. Mutations in the extracellular proteins ADAMTSL2 or fibrillin-1 lead to recessive or dominant GD, respectively. Excess TGFβ signaling was described in cells derived from GD patients and may constitute a major pathogenetic mechanism. ADAMTSL2 directly interacts with latent transforming growth factor-β binding protein (LTBP)-1 and fibrillin-1. Fibrillin-1 and -2 assemble into microfibrils and modulate extracellular TGFβ and BMP signaling. The *Adamtsl2* gene was deleted in mice by an intragenic IRES-*lacZ-neomycin* cassette, which also provided an expression reporter. *Adamtsl2* deletion resulted in neonatal lethality due to an inter-ventricular septal defect (VSD) and lung abnormalities. β -gal reporter staining showed *Adamtsl2* expression within the embryonic heart and lung, consistent with the observed anomalies. Cardiac expression was primarily localized to the crest of the inter-ventricular septum, the atrio-ventricular valve annulus and coronary vessels. Histology revealed a VSD in 80% of the mice with enhanced, localized fibrillin-1 and -2 staining at the crests of the inter-ventricular septum. In vitro, recombinant ADAMTSL2 bound to both fibrillin-1 and -2, and accelerated the biogenesis of fibrillin-1 microfibril formation in fetal bovine nuchal ligament cells, while blocking fibrillin-2 assembly. This suggests that ADAMTSL2 may provide a mechanism to actively regulate the ratio of fibrillin-1 to fibrillin-2 in microfibrils, with potential consequences for TGFβ/BMP signaling. These studies suggest a new role for extracellular matrix in regulation of cardiac development.

S1.2 Complex trait analysis of ventricular septal defects caused by *Nkx2-5* mutation

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Congenital heart disease is a complex trait. The same deleterious mutation typically causes widely varying presentations because additional, poorly defined factors modify phenotype. Historically, the modifiers have received less scrutiny than the causes. Nevertheless, the factors, especially ones that reduce risk, may suggest preventive strategies that a focus on monogenic causes has not. Hence, we assessed the role of genetic and environmental factors on the incidence of ventricular septal defects (VSD) caused by an *Nkx2-5* heterozygous knockout mutation. We phenotyped >3100 hearts from a second generation intercross of the inbred mouse strains C57BL/6 and FVB/N. Genetic linkage analysis mapped loci with LOD scores of 5-7 on chromosomes 6, 8 and 10 that influence the susceptibility to membranous VSD in *Nkx2-5*$^{+/-}$ animals. The chromosome 6 locus overlaps one for muscular VSD susceptibility. Multiple logistic regression analysis for environmental variables revealed that maternal age is correlated with the risk of membranous and muscular VSD in *Nkx2-5*$^{+/-}$ but not wild-type animals. The maternal age effect is unrelated to aneuploidy or a genetic polymorphism in the progeny. Ovarian transplant experiments between young and old females indicate that the basis of the maternal age effect resides in the mother and not the oocyte. Experiments to characterize a potential metabolic basis of the effect strongly suggest that the VSDs caused by *Nkx2-5* mutation can be prevented. Enumerable factors contribute to the presentation of a congenital heart defect. Their characterization in a mouse model offers the opportunity to define unanticipated pathways and to develop strategies for prevention.

S1.3 Functional analysis of novel ZIC3 mutations identified in patients with heterotaxy

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Loss of function mutations in the zinc finger in cerebellum 3 (ZIC3) transcription factor result in heterotaxy, a condition characterized by abnormal left-right positioning of thoraco-abdominal organs and a wide variety of congenital anomalies, particularly of the cardiovascular system. Mutations in *ZIC3* have been reported in approximately 75% of all familial and 1% of all sporadic heterotaxy cases and have been associated with

VACTERL, a constellation of malformations phenotypically overlapping heterotaxy. The presence of a polyalanine (polyA) expansion in one patient with VACTERL is of particular interest as pathogenic expansions have been identified in several other developmentally critical transcription factors, including *ZIC2.* To further define the incidence and functional significance of *ZIC3* mutations in heterotaxy, coding regions and splice junctions were screened in 200 unrelated heterotaxy patients. Nine mutations (8 novel) were identified, including a single alanine expansion (c.insGCC159-160). Functional analyses were supplemented by 4 additional recently reported *ZIC3* mutations. Aberrant ZIC3 cytoplasmic localization was observed for mutations spanning multiple nuclear localization domains between amino acids 155 and 318 and correlated with decreased transactivation of a luciferase reporter. A missense mutation within zinc finger 5 (p.A447G) surprisingly increased luciferase transactivation, despite elevated levels of cytoplasmic ZIC3. Neither polyA tract expansion differed significantly from wildtype with respect to either luciferase transactivation or ZIC3 subcellular localization. These analyses collectively indicate a higher than expected percentage of *ZIC3* mutations in patients with sporadic heterotaxy and suggest alternative pathogenesis of some *ZIC3* mutations, notably those within the polyA tract.

S1.4 The Genetic Basis of Isolated Tetralogy of Fallot

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Background: Tetralogy of Fallot (TOF) represents 10% of congenital heart disease, which are the most common birth defect in human. The majority of TOF are isolated non-syndromic cases of unknown precise cause, which applies for the majority of congenital heart disease. *Methods:* We performed targeted resequencing of over 1,000 genes and microRNAs as well as whole transcriptome and miRNome analysis in TOF cases, parents and controls using next-generation sequencing techniques. We defined a set of TOF genes with deleterious variations and which are mutated at a higher rate in TOF subjects compared to healthy controls. *Results:* We identified an oligogenic architecture underlying TOF, which discriminate TOF cases from controls. On average, TOF subjects show a combination of novel and inherited variations in five genes. The majority of genes has known association with human cardiac disease and/or shows a cardiac phenotype in mouse mutants. Seven genes are novel and have not yet been linked to a cardiac phenotype. Functionally interacting yet individual mutations lead to the same phenotypic outcome during development. The majority of TOF genes shows continuous relevance during adulthood. *Conclusions:* Isolated TOF is a genetic disorder involving multiple genes. Although subjects show a range of individual mutations, the phenotypic outcome is similar because TOF genes show common patterns of functional interactions. Sequencing approaches can help to define a genetic risk profile for families that can also be used to define differences in the long-term clinical outcome, which should permit a personalization of diagnostic and therapeutic strategies.

S1.5 An Excess of Deleterious Variants in VEGF-A Pathway Genes in Down Syndrome-Associated Atrioventricular Septal Defects

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About half of people with trisomy 21 have a congenital heart defect (CHD) while the remainder have a structurally normal heart, demonstrating that trisomy 21 is a significant risk factor but is not causal for abnormal heart development. We used a candidate gene approach in a study cohort of individuals with Down syndrome (DS) to determine if rare genetic variants in genes involved in atrioventricular valvuloseptal morphogenesis contribute to atrioventricular septal defects (AVSD) in this sensitized population. We found a significant excess (p<0.0001) of variants predicted to be deleterious in DS with AVSD cases compared to DS with no heart defect controls. The variants with the highest probability of being damaging were found in six genes: *COL6A1*, *COL6A2*, *CRELD1*, *FBLN2*, *FRZB* and *GATA5*. Several of the variants were recurrent in unrelated cases. There were no variants with an equal probablity of being damaging in these genes found in controls, demonstrating a highly specific association with AVSD. Of note, all of these genes are in the VEGF-A pathway even though the candidate genes analyzed in this study represented numerous biochemical and developmental pathways, suggesting that rare variants in the VEGF-A pathway may contribute to the genetic underpinnings of AVSD in humans.

S1.6 Identification of Novel Mutations in a Large Family with Different Forms of Congenital Heart Disease

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Background: Congenital Heart Disease (CHD) affects more than 1% of newborns and is widely believed to be due to mutations in genes involved in cardiac development. However, to date only about 5% of the genetic causes of CHD is known. Objective: Our objective is to study families with CHD from Lebanon, a highly consanguineous population, using a combination of Sanger and Next-Generation sequencing, as well as SNP genotyping. Methods: Target-capture sequencing of cardiac-enriched genes was performed on more than 150 families with CHD. The phenotypes include septal defects (atrial and ventricular), valvular disease (pulmonary stenosis, aortic stenosis, tricuspid atresia, bicuspid aortic valve), coarctation of the aorta, tetralogy of fallot, transposition of the great arteries, single ventricle, Ebstein anomaly, and atrioventricular canal defects. Mutation-negative large multiplex families were studied using SNP genotyping and whole-exome sequencing. Results: Target-capture sequencing analysis of 50 families identified several homozygous missense mutations in cardiac genes (JAG1, NOTCH1, MID1, NF1, NUP188, NFATc4, FLNA). Mutation segregation with the phenotype was confirmed in most of the families. Analysis of a particular large family with different forms of congenital diseases point out to a multi-factorial mode of inheritance involving Hand2, NFATC4, and Nephrin. Conclusion: Dominant mutations occur in consanguineous populations. The combination of SNP genotyping and next-generation sequencing is an expected method to study gene mutations in families with congenital heart disease, particularly those with recessive mutations.

1.7 Novel role of altered cardiac function in the progression of heart defects in Fetal Alcohol Syndrome (FAS)

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Over 500,000 American women per year report drinking alcohol during pregnancy, with 1 in 5 who also binge drink. Even low levels of prenatal alcohol/ethanol exposure can produce birth defects in humans. Epidemiological studies suggest that 54% of live-born children with Fetal Alcohol Syndrome (FAS) present with cardiac anomalies. While the mechanisms of ethanol exposure have been studied extensively, most studies fail to consider the role of altered cardiac function in producing congenital heart defects. It is already known that changes in hemodynamics can profoundly affect cardiac development. We hypothesized that ethanol exposure creates early hemodynamic anomalies which contribute significantly to subsequent cardiac structural and functional defects. We employed optical coherence tomography (OCT), which is a non-destructive imaging modality capable of real-time, micrometer-scale resolution imaging. OCT allowed us to accurately map changes in hemodynamic forces (e.g. shear stress) and the resultant structural abnormalities in the live embryo at very early stages, when the trajectory to heart defects can begin. In our studies, avian embryos exposed to ethanol during gastrulation exhibited alterations in overall embryo body flexure, blood flow, shear stress and cardiac cushion development during heart looping stages. A combination of myo-inositol (MI) and folate (FA) given at gastrulation stages are known to prevent ethanol-induced cardiac abnormalities in mouse/avian models. In

future studies, we will test whether FA/MI supplementation rescues these defects through normalization of cardiac function. Our contributions could be a first step in implementing new therapeutic strategies based on FA/MI prevention of birth defects. Research Support: HL083048, HL095717.

1.8 Noonan syndrome associated RAF1 mutant evokes hypertrophic cardiomyopathy features in human cardiomyocytes in vitro.

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Noonan syndrome (NS) and its related disorders, which are now called “RASopathies”, are caused by aberrant activation of RAS/ERK pathway. Most RASopathies feature proportional short stature, facial dysmorphia, cognitive impairment and cardiac defects. The cardiac manifestations in RASopathies vary widely, but hypertrophic cardiomyopathy (HCM) is found in virtually all NS cases caused by RAF1 allele that encode a hyperactive kinase mutant. HCM also is common in LEOPARD syndrome and Costello syndrome. We reported previously that a mouse model of NS caused by a kinase-activated Raf1 mutant recapitulates major features of NS, including HCM. Importantly, these features were normalized by post-natal treatment of MEK inhibitor. To extend these mouse studies to pre-clinical human models to better identify detail molecular basis and signaling as well as to aid in the development of new therapies for RASopathies, we have generated human induced pluripotent stem cells (hiPSCs) from fibroblasts of multiple RASopathy patients. We found that a kinase-activating RAF1 mutant causes increased cell size of cardiomyocytes differentiated from hiPSCs compared with normal hiPSCs. We also found increased calcium sensitivity and lower response to b adrenergic stimulation when we cultured differentiated cardiomyocytes as engineered heart tissue, which allows us to apply mechanical forces on these cardiomyocytes. Our data show that NS associated RAF1 mutant can cause HCM phenotypes in vitro and provide a potential pre-clinical system for testing new therapies.

1.9 Engineering new mouse models to map dosage-sensitive genes in Down syndrome congenital heart defects

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Trisomy of human chromosome 21 (Hsa21) occurs in ~1 in 750 live births and the resulting gene dosage imbalance causes Down syndrome (DS). One of the most important medical aspects are the congenital heart defects (CHD), which affect around half of all individuals with DS. The defects observed in DS range from simple chamber septation to complex CHD involving multiple aspects of the heart anatomy. The most common CHD in DS affect the atrio-ventricular (AV) junction. Using high-resolution episcopic microscopy (HREM) in mouse embryonic hearts, we have studied a ‘transchromosomic’ (Tc1) mouse model of DS, which carries Hsa21 as a freely segregating chromosome. The Tc1 mouse closely resembles human DS CHD, replicating many of the features of the AV septal defects found in DS (Dunlevy, 2010). To unravel the mechanisms of defective heart formation in the Tc1 mouse embryo, we are using lineage markers to study the tissues involved in development of the AV junction. In order to identify dosage-sensitive genes that may underlie DS CHD and other complex biological effects of trisomy, we are generating a high-resolution mapping panel of mouse strains with partial trisomies and monosomies for regions of mouse chromosomes orthologous to Hsa21. Using systematic HREM analysis, we are examining partial trisomic strains to identify regions that are sufficient to induce the CHD phenotype. Conversely, by crossing Tc1 mice to partial monosomic strains and assaying the effect of each interval on the cardiac phenotype, we are testing individual genes for their potential role in DS CHD.

1.10 Recovery of ENU induced mutations causing congenital heart disease using next-gen sequencing

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Forward genetic screens provide a powerful non-gene biased approach to elucidate the genetic basis for congenital heart disease (CHD). In the past this has been hampered by difficulties in mutation recovery. In our current large-scale recessive mouse ENU mutagenesis screen, we are pursuing the use of whole genome/whole exome sequencing for mutation recovery. To evaluate the efficacy of our mutation recovery

pipeline, the mutant Destro exhibiting complex CHD with heterotaxy was analyzed by both whole exome and whole genome sequencing. A multi-step bioinformatics filtering strategy was developed for mutation recovery. First, we removed sequence variants present in dbSNP129 or our in-house mouse databases. Second, coding variants likely to be homozygous based on sequence coverage, unique reads, and strandedness were prioritized as potential candidates. Third, mutations are further rank ordered based on cross-species conservation. Finally, the causal mutation is identified by genotyping multiple mutants with the same phenotype. Whole-genome sequencing of Destro with 8x coverage together with our filtering strategy reduced 178,848 variants to just 6, with genotyping analysis identifying Bicc1$^{c.606+2T>C}$ as the disease-causing mutation. This same mutation was also recovered by whole-exome sequencing with ~50x target-coverage. In another mutant line 370 with coarctation and diaphragmatic hernia, exome sequencing analysis yielded a single mutation in Lox1, which was confirmed to be the disease causing mutation. Over 10 mutations have been recovered thus far. These results suggest our whole exome-sequencing pipeline will provide a cost-effective strategy for meeting our goal of pursuing a saturation level mutagenesis screen to recover mutations causing CHD. Supported by funding from U01-HL098180

1.11 Resource Sharing by the Cardiovascular Development Consortium: A basic research component of the NHLBI Bench to Bassinet Program

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The NHLBI Cardiovascular Development Consortium (CvDC) is a collaborative association of four Research Centers that comprise the basic science component of the NHLBI Bench-to-Bassinet (B2B) Program. The roles of the CvDC within the B2B Program are two-fold; 1) to expand knowledge of the complex regulatory networks that affect cardiac development, and 2) through interaction with a sister consortium within the B2B Program, the Pediatric Cardiac Genomics Consortium, apply CvDC discoveries to clinically-relevant genomics studies of human congenital heart disease. The role of the CvDC within the cardiovascular development community is to generate and rapidly share novel resources, including published and unpublished datasets, novel model organisms, reagents, and tools. As CvDC resources become available, they are assembled into an online collection accessible to the scientific community through a portal on the B2B Program website (http://www.benchtobassinet.org/Investigators.asp). The collection includes resources directed to the study of the epigenetics of cardiac development, gene discovery of genetic variants and networks causative of heart defects in animal models, and transcriptome changes in response to genetic variants or epigenetic regulation. The portal provides descriptions of available resources, and links to data repositories or vendors. Data and bioinformatics tools, such as mouse and zebrafish ChIP-seq and RNA-seq datasets or completeMOTIFs, are accessible through the CvDC data repository (https://b2b.hci.utah.edu/gnomex/gnomexGuestFlex.jsp), while mouse models are available through Jackson Laboratories or directly from CvDC Research Centers. Alternatively, CvDC datasets can be searched or browsed directly on the data repository, and mouse lines on the Jackson Laboratories Mouse Genome Informatics website.

1.12 Large scale forward genetic screen in fetal mice reveals genetic etiology for hypoplastic left heart syndrome and wide spectrum of congenital heart defects

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We are conducting a large-scale mouse ENU mutagenesis screen using noninvasive mouse fetal echocardiography to recover mutations causing congenital heart disease (CHD). Ultrasound screening of 42,628 mouse fetuses yielded 2,430 abnormal fetuses. Of these, 1,614 (66.4%) exhibited CHD, with 43.5% dying prenatally. Growth retardation was highly associated with cardiac defects (88.3%). Over 80 mutant lines with heritable CHD phenotypes have been recovered; the most common being ventricular septal defect (VSD),

found in 59 mutant lines. Double outlet right ventricle and atrioventricular septal defect were the most common complex CHD phenotypes observed. Surprisingly, 27 of the mutant lines exhibited laterality defects. We also recovered three mutant lines with hypoplastic left heart syndrome (HLHS), a phenotype not previously observed in any mouse models. Ultrasound phenotyping has high detection sensitivity for hypoplastic left and right heart syndromes, VSD, and persistent tru ncus arteriosus/pulmonary atresia, but low detection rate for coronary fistula and aortic arch anomalies. However, mutants with such defects can be recovered using micro-computed tomography as a secondary screening tool. Exome sequencing is underway to recover the disease causing mutations, with mutations identified in 11 mutant lines thus far. Lines with heritable CHD phenotypes have been cryopreserved at the Jackson Laboratory for public access. These lines are annotated in the Mouse Genome Informatics (MGI) database with detailed phenotype information provided in multiple imaging modalities. Clinical CHD Fyler codes are also curated to show human CHD correlates. These novel mouse models will greatly aid in elucidating the developmental etiology of HLHS and other CHD.
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1.13 Molecular genetic investigation of Left Ventricular Outflow Tract Obstruction

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Introduction: Left ventricular outflow tract obstruction (LVOTO) is a significant cause of morbidity and mortality and a strong genetic component is established. A patient has been identified with LVOTO, and a balanced translocation with breakpoints on 14q and 15q. This is suggestive of a disease causing gene within either region. Hypothesis: A gene within 14q or 15q is critical to cardiogenesis and mutations within it are a cause of LVOTO in humans. Methods: Using mouse RNA Whole Mount In Situ Hybridisation, the embryonic expression pattern of murine orthologous genes within the 1 megabase region of the breakpoints were studied in developing mouse embryos to determine expression during cardiogenesis. Since genes involved in mammalian cardiogenesis are conserved, murine orthologs of human genes can usefully be screened in this way. Images were recorded using optical projection tomography. The expression patterns allowed prioritisation of genes to be selected for immunohistochemistry and sequencing. Results: Literature review for cases of 14q and 15q deletions and the associated phenotypes identified a region on 15q as the most likely locus. WISH shows expression of MCTP2 (15q26.2) in mouse embryonic hearts and using immunohistochemistry, MCTP2 was also found to localise to the sarcomeres of cardiomyocytes in human fetal hearts. Sequencing of MCTP2 in 53 cases of LVOTO looking for mutations within the coding sequence/splice sites revealed a missense mutation, p.Arg340Cys not found in 310 ethnically matched control chromosomes. Conclusions: MCTP2 is a good candidate disease gene for LVOTO.

1.14 Exome Sequencing Analysis of a Pleiotropic Congenital Heart Disease Family

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Background – Non-syndromic congenital heart defects (CHD) have been observed in families underscoring possible single and multiple gene defects that are yet to be completely identified. Candidate gene and other approaches have limited sensitivity in detecting rare causal variants. We used next generation sequencing to identify disease-associated variants in a family with highly penetrant pleiotropic CHD. ***Methods and Results –*** Whole exomic sequencing performed on 4 members of a family with different CHD lesions using Illumina Hi-Seq 2000 platform, yielded more than 1700 variants. After excluding common variants (1000 genomes, dbSNP) and sequencing artifacts, variants were prioritized based on cardiac and vascular expression of genes. Of the 55 novel and rare variants (including single nucleotide variations and indels) seen in the 4 members, 49 were non-synonymous, 3 were predicted to alter splicing, and 3 resulted in a frameshift. Further prioritization reduced the number of variants potentially involved in CHD to 5. Of those 5, a novel non-synonymous variant on chromosome 4 segregated in affected family members i.e. the proband with interupted aortic arch, proband's brother with ventricular septal defect, father with patent ductus arteriosus, but not in mother with no heart disease. The mutation has a good SIFT prediction of 0.01. Mice knockouts have previously shown this gene is essential for embryonic hematopoiesis including heart development. ***Conclusions –*** Next-generation

sequencing can help identify novel and rare variants associated with CHD. We will study the expression of this gene in cardiac tissue available in the proband and replicate in additional CHD families.

1.15 High prevalence of left-right patterning mutants recovered in a large scale mouse mutagenesis screen for mutations causing congenital heart defects

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Some of the most complex congenital heart disease (CHD) is found in conjunction with heterotaxy (HTX), the randomized left-right patterning of visceral organ situs. In our ongoing large scale mouse mutagenesis screen for mutations causing congenital heart disease, more than 1/3 of the mutant lines recovered from our screen exhibited left-right patterning defects. This would suggest an important role for pathways involved in left-right patterning in cardiovascular morphogenesis, perhaps not unexpected given the heart is the most left-right asymmetric organ in the body. From over 80 mutant lines recovered thus far in our screen, 27 display laterality defects (37%). Of these, 14 exhibit either complete mirror symmetric visceral organ situs (situs inversus totalis; SIT) or HTX, 10 lines displaying only HTX, and 3 lines with only SIT. Importantly, heterotaxy mutants almost always have complex CHD, but complex CHD is never observed in mutants with SIT. In mutants where the mut ations have been identified, genotyping have revealed some mutant animals have normal situs (situs solitus) and such animals never have complex CHD. Given the importance of motile cilia function in laterality specification, we also carried out videomicroscopy of tracheal airway cilia as a proxy for nodal cilia motility in mutant lines exhibiting laterality defects. We have found mutants exhibiting a wide range of ciliary phenotypes ranging from normal cilia motility, to those with slow and dyskinetic ciliary beat, hyperkinetic ciliary beat, or immotile cilia. Interestingly, as we have reported previously, mutants with immotile cilia can also generate mutants with normal situs solitus or SIT, suggesting motile cilia function may be dispensible for left-right patterning. By systematically categorizing the situs defect and CHD phenotypes observed in laterality mutants recovered in our mutagenesis screen, we hope to ascertain the discrete steps involved in visceral organ situs specification, and elucidate the role of cilia in left-right patterning and cardiac morphogenesis. Supported by U01-HL098180.

1.16 The National Registry of Genetically Triggered Thoracic Aortic Aneurysms (GenTAC): Registry Progress and Research Successes

Cheryl L. Maslen, PhD, on behalf of the GenTAC Consortium

Funded by the National Institutes of Health, the GenTAC Registry is a multicenter, longitudinal, observational cohort study of patients with conditions involving genetically triggered thoracic aortic aneurysm and/or dissection (TAAD). GenTAC was established to provide a biospecimen inventory and bioinformatics infrastructure that will enable research to advance the clinical management of such patients. Primary diagnoses include Marfan syndrome, bicuspid valve with aneurysm and/or a family history of aneurysm, idiopathic TAAD in patients < 50 years of age, Turner syndrome, familial TAAD, other congenital heart disease with TAAD, Ehlers-Danlos syndrome, and Loeys-Dietz syndrome. To date GenTAC has recruited 2826 subjects, with a final goal of 4000 registrants. Initial analyses of GenTAC data/specimens have included studies of genetic causes for aortic syndromes via gene sequencing, SNP, CNV and genome-wide association studies, potential usefulness of TGF-b blood levels as prognostic or therapeutic marker in Marfan subjects, surgical approaches/outcomes for ascending aortic conditions and gender differences in TAAD. Other studies in progress include cross-sectional and longitudinal data regarding phenotype-genotype correlations of disease risk factors, features, treatment, and outcomes, and analysis of imaging methods/integration of imaging findings with clinical and genetic data. GenTAC phenotyping and imaging cores have been established to facilitate these inquiries. The GenTAC Registry is a resource for anyone in the scientific community interested in advancing our understanding of genetically mediated TAAs and their causes, diagnosis, and optimal treatment. Investigators interested in utilizing GenTAC for ancillary studies should contact the registry at gentac-registry@rti.org or apply at http://gentac.rti.org.

1.17 High-throughput phenotyping for structural heart defects in fetal/neonatal mice using micro-computed tomography

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We are conducting a high-throughput mouse mutagenesis screen to recover mutations causing congenital heart defects (CHD) using fetal echocardiography. Mutant fetuses identified to have cardiac defects by ultrasound must be further phenotyped by necropsy and histological analysis for CHD diagnosis. In this study, we investigated the utility of micro-computed tomography (micro-CT) as a high-throughput screening tool for identifying structural heart defects in fetal/newborn mice. Micro-CT scanning of 2105 fetal/newborn mice carried out with iodine contrast agent revealed a spectrum of CHD, the most common being ventricular septal defects (VSDs; 14.58%), followed by outflow tract anomalies (2.6%), including transposition of the great arteries (TGA; 0.67%), double outlet right ventricle (DORV; 1.71%), pulmonary atresia (PA; 0.10%), and persistent truncus arteriosus (0.14%). We also observed right aortic arch (RAA; 1.33%), coarctation/interrupted aortic arch (0.57%), endocardial cushion defects (1.05%) and tricuspid atresia/stenosis (0.62%). These same specimens were evaluated by necropsy, with some further analyzed by episcopic fluorescence image capture (EFIC). EFIC generates serial 2D histological image stacks that can be digitally re-sliced in any orientation for CHD assessment. Necropsy and EFIC imaging confirmed all RAA diagnoses (n=15/15), 77.4% of VSDs (n=72/93), and 91.2% of TGA/DORV/PA (n=31/34). We observed false positive rates of 17.2% (n=16/93) for VSDs and 5.9% (n=2/34) for TGA/DORV, and a false negative rate of 5.4% (n=5.93) for VSDs. Overall, these findings indicate micro-CT is an invaluable tool for rapid screening and detection of a wide spectrum of CHD, the main limitation being the time required for image processing and diagnostic evaluation. Supported by U01-HL098180.

1.18 Identification of Genetic Modifiers of Congenital Heart Defects

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Congenital heart defects (CHD) are the most common congenital anomaly in live births. There is an especially high incidence of CHD in Down syndrome (DS), where 40 – 50% of affected individuals have a CHD. Thus, people with trisomy 21 are sensitized to CHD, but the fact that half of those with DS have a normal heart suggests that additional genetic and environmental factors interact with trisomy 21 to disrupt heart development. We have established a study to identify genetic risk factors for CHD in the sensitized DS population, genes whose individual contributions are too small to be identified on a euploid background (the DS Heart project, http://inertia.bs.jhmi.edu/ds/index.html). Here we used the Ts65Dn mouse model of DS as a corresponding sensitized population to study the role of the *Tbx5* gene in CHD. Mutations of *Tbx5* are associated with abnormalities of the heart and upper limbs. A *Tbx5* null allele was crossed into Ts65Dn mice and newborn pups were sacrificed and examined for CHDs. We saw a significant difference between trisomic and euploid pups in the frequency of two particular defects, overriding aorta and an unusual connection between the right atrium and left ventricle. Almost 50% of the trisomic $Tbx5^{+/-}$ mice present with overriding aorta and a ventricular septal defect, while less than 20% of the euploid pups have this defect. These results suggest that there is an interaction between *Tbx5* and trisomy affecting heart development. Published studies with Tbx5 have suggested several genes as participants in this interaction. Future study will involve investigation into these gene(s) and the mechanisms behind this interaction.

1.19 Confocal episcopic fluorescence image capture for phenotyping complex congenital heart defects and other structural malformations

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We have previously shown episcopic fluorescence image capture (EFIC) is a powerful imaging tool for phenotyping complex structural heart defects. With EFIC imaging, paraffin embedded tissue is sectioned with a sledge microtome, and tissue autofluorescence at the block face is captured by epifluorescence with each successive cut to generate registered 2D serial image stacks suitable for 2D digital reslicing or rapid 3D reconstructions. This allows complete diagnosis of even the most complex structural heart malformations. For standard EFIC imaging, light-blocking dye is added to the paraffin to prevent bleed through fluorescence from deeper tissue layers, a tedious and difficult process requiring superheating the wax. To overcome this requirement, we adapted EFIC imaging to use the Leica LSI confocal macroscope for confocal enhanced EFIC

(CEFIC) imaging of standard paraffin embedded tissue. Using CEFIC, we characterized the congenital heart defects (CHD) in 435 fetal/newborn mice from 115 mutant mouse lines. A wide spectrum of CHD were observed, including ventricular septal defects (52%), ventricular hypertrophy (20.9%), ventricular non-compaction (17.5%), double outlet right ventricle (14.5%), overriding aorta (9%), transposition of the great arteries (3.7%), aortic stenosis (5.7%), pulmonary stenosis (4.1%), coronary fistulas (5.1%), major aortopulmonary collateral arteries (MAPCAs), interrupted aorta (2.5%), double aortic arches/vascular rings (2.1%), and hypoplastic left (0.7%) or right ventricles (0.9%). CEFIC can also identify other malformations difficult to ascertain by standard histology, such as tracheoesophageal fistulas and cloacal septation defects. We further showed CEFIC can be combined with fluorescence markers and also used for phenotyping other animals including the zebrafish.

1.20 Mechanisms of Folate Protection of Cardiac Embryogenesis

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We demonstrated that folate (FA) prevents cardiac birth defects acutely induced during gastrulation on embryonic day (ED) 6.75 by a binge level of alcohol, by the drug lithium (Li) or by homocysteine (HCy). Despite FA fortification, the mechanism of FA protection remains unknown. This study was to identify genes in the ED 15.5 affected cardiac outflow tract (OFT) that are altered by maternal embryonic exposure to the above factors on ED 6.75, with and without FA supplementation. We used Affymetrix microarray analyses of gene expression changes in the ED 15.5 embryonic OFT tissues. Validation of specific gene expression was carried out on ED 7.5 and again at ED 15.5, the latter in embryos displaying abnormal cardiac function as monitored by echocardiography. In the Li- affected OFT, 51 genes associated with Wnt and phosphatidylinositol signaling were down-regulated; 21 genes were up-regulated. Sixteen Wnt related genes were down-regulated by both Li and HCy; 23 were upregulated. A number of the genes overlapped. Thirty-eight genes that were misexpressed by Li and reversed by FA related to genes associated with chromatin modification reactions and with methylation. Key gene expression changes in the above pathways have been validated by RT-PCR and in situ hybridization. Taken together, these results confirm that Li, HCy and alcohol misexpress genes in Wnt/PtIn signaling and in FA metabolism that can lead to methylation and chromosome modification changes. Early supplementation with FA initiated right after conception protects one carbon metabolism, methylation reactions and gene expression leading to normal cardiogenesis.

1.21 Value of high-throughput mutagenesis screening with fetal echocardiography for recovery of ENU-induced mouse mutations causing congenital heart disease

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Compared to micro-computed tomography (micro-CT), episcopic fluorescence image capturing (EFIC), ultrasound has such disadvantage as lower spatial resolution, but with rapid, inexpensive and noninvasive, it is used for interrogating live pregnant mice of congenital heart disease (CHD). In the study, to improve the efficacy of ethylnitrosourea (ENU) mutagenesis high-throughput screening, we established a strategy of fetal echocardiography. First, fetal mice were rapidly screened using high frequency (15MHz) ultrasound (Acuson C512). Then, pregnant females with interesting fetuses were scanned carefully using ultra-high frequency (30~40MHz) ultrasound biomicroscopy (VEVO2100) with low penetration and limited width and depth range. With the strategy, 20,228 fetuses were screened between embryonic days 13.5 and 18.5 in utero, 1125(5.6%) showed developmental anomalies, with 683(60.7%) exhibiting cardiovascular defects, of which 125(24.4%) died prenatally at follow-up. There were 2915 fetuses further scanned carefully with ultrasound biomicroscopy, 530(18.2%) showed developmental anomalies, with 390(73.6%) exhibiting a wide spectrum of CHD, including atrioventricular septal defects (15), conotruncal anomalies (43) (double outlet right ventricle, persistence truncus arteriosus, transposition of the great arteries, etc), laterality defects (20) (CHD with heterotaxy and situs inversus totalis), ectopiacordis (2), etc. In addition, growth retardation, hydrops, craniofacial/limb anomalies and body wall defects were highly associated with CHD. The results were confirmed by further anatomical analysis of Necropsy, micro-CT, EFIC in abnormal pups harvested immediately or euthanized after birth. In conclusion, ultrasound high-throughput screening with biomicroscopic scanning could play an essential role in

cardiovascular phenotyping, and improve greatly the efficacy to recover ENU-induced mouse mutations causing CHD. (Supported by U01-HL098180)

1.22 Identification of pathogenic copy number variations in syndromic CHDs by SNP array

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High resolution SNP array has provided new opportunities to identify genetic lesions associated with human diseases such as congenital heart defects (CHDs). We screened a cohort of patients with syndromic congenital heart defects (CHD with extra-cardiac phenotypes) for pathogenic Copy Number Variations (pCNV) by SNP-array and detected genomic lesions in 27 out of 180 (15%) patients. 78% of the affected individuals (21/27) have an aberrant chromosomal region containing a gene previously known to be associated with CHDs. Six patients had novel CNVs that contain candidate genes involved in cardiogenesis. Specially, two CNVs with the 3p26.1-26.3del/10p15.3-15.1dup were found in a heterozygous twin with complex CHD. Interestingly, a candidate gene for ventricular aneurysms and septal defects was recently mapped in the genomic region 10p15 [Tremblay et al, Eur Heart J. 2011, 32 (5):568-73], which overlaps with the genomic region containing the10p15.3-15.1dup. Further analysis of these CNVs and candidate genes identified in these CNVs will be presented.

1.23 Mutations in the T (brachyury) gene cause a novel syndrome consisting of sacral agenesia, defective ossification of the vertebral bodies and persistent notochord

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The T gene (Brachyury gene) is the founding member of the T-box family of transcription factors. The protein encoded by this gene is an embryonic nuclear transcription factor that binds to specific DNA elements and effects transcription of genes required for mesoderm formation and differentiation. The protein is localized to notochord-derived cells and is vital for the formation and differentiation of the posterior mesoderm and axial development of all vertebrates. We report here on four patients from three consanguineous families exhibiting sacral agenesia, delayed ossification of the vertebral bodies and a persistent notochord, the heart was unaffected. Homozygosity mapping identified a common 4.1Mb homozygous region on chromosome 6q27, containing the T gene. Sequencing of T in the affected individuals led to the identification of a homozygous mutation, p.H171R, in the highly conserved T-box and absent from control alleles. The homozygous mutation results in diminished DNA binding, interferes with bone morphogenetic protein (BMP) signaling and causes changes in expression of genes involved in notochord maintenance and chondrogenesis. In conclusion, we present evidence that a novel syndrome consisting of sacral agenesia, defective ossification of the vertebral bodies and persistent notochord is caused by a homozygous mutation in the T gene. The mutation leads to a moderate loss of function on DNA binding and disturbed expression of important downstream targets genes involved in proper posterior mesoderm and axial development, likely resulting in the unique phenotype.

1.24 TWO COMPOUND HETEROZYGOUS MUTATIONS in NFATC1 in a LEBANESE PATIENT with TRICUSPID ATRESIA

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Tricuspid Atresia (TA) is a rare form of congenital heart disease (CHD) with usually poor prognosis in humans. It presents as a complete absence of the right atrio-ventricular connection secured normally by the tricuspid valve. Defects in the tricuspid valve are so far not associated with any genetic locus, although mutations in numerous genes were linked to multiple forms of congenital heart disease. In the last decade, Knock-out mice have

offered models for cardiologists and geneticists to study the causes of congenital disease. One such model was the Nfatc1-/- mice embryos which die at mid-gestation stage due to a complete absence of the valves. NFATC1 belongs to the Rel family of transcription factors members of which were shown to be implicated in gene activation, cell differentiation, and organogenesis. We have previously shown that a tandem repeat in the intronic region of NFATC1 is associated with ventricular septal defects. In this report, we unravel for the first time a direct link between a mutation in NFATC1 and TA. Two heterozygous missense mutations were found in the NFATC1 gene in one indexed-case out of 19 patients with TA. The two amino-acids changes were not found in other patients with CHDs nor in the control healthy population. Moreover, we showed that these mutations alter dramatically the normal function of the protein at the localization, DNA binding and transcriptional levels suggesting they are disease-causing.

1.25 Actin Mutation that Causes Patent Ductus Arteriosus Alters Regulation by Formin

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More than thirty mutations in ACTA2, which encodes α-smooth muscle actin, have been identified to cause autosomal dominant cardiovascular disease. All mutations cause thoracic aortic aneurysm but the R256H mutation is of interest because it distinctively causes patent ductus arteriosus. R256H is one of the more prevalent mutations and, based on its molecular location near the strand-strand interface in the actin filament, may affect F-actin stability. To understand the molecular ramifications of the R256H mutation, we generated Saccharomyces cerevisiae yeast cells expressing only R256H yeast actin as a model system. These cells displayed abnormal cytoskeletal morphology and increased sensitivity to Latrunculin A. After cable disassembly induced by transient exposure to Latrunculin-A, mutant cells were delayed in reestablishing the actin cytoskeleton. In vitro, mutant actin exhibited a higher than normal critical concentration and a delayed nucleation. Consequently, we investigated regulation of mutant actin by formin, a potent facilitator of nucleation and a protein needed for normal vascular smooth muscle cell development. Mutant actin polymerization was inhibited by the FH1-FH2 fragment of the yeast formin, Bni1. This fragment strongly capped the filament rather than facilitating polymerization. Interestingly, phalloidin or the presence of wild type actin reversed the strong capping behavior of Bni1. Together, the data suggest that the R256H actin mutation alters filament conformation resulting in instability and misregulation by formin. These biochemical effects may contribute to inappropriate persistence of the ductus arteriosus.

1.26 Perturbation of the titin/MURF1 signaling complex is associated with hypertrophic cardiomyopathy in a fish model and in human patients

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Background: Hypertrophic cardiomyopathy (HCM) is a hereditary disease characterized by cardiac hypertrophy with diastolic dysfunction. Gene mutations causing HCM have been found in about half of the patients, while pathogenesis remain unknown for many cases of HCM. To identify novel mechanisms underlying HCM pathogenesis, we performed forward-genetic N-ethyl-N-nitrosourea (ENU) mutagenesis screening. Methods and Results: We generated a cardiovascular-mutant medaka fish non-spring heart (nsh), which showed diastolic dysfunction and hypertrophic myocardium. The nsh mutant heart lost elasticity and beat in a rigid manner, as determined by high-speed video imaging with the analysis using motion vector prediction (MVP) method. The nsh homozygotes had disrupted sarcomeres and expressed pathologically stiffer titin isoforms. In addition, the nsh heterozygotes showed M-line disassembly that is similar to the pathological changes found in HCM. By

positional cloning, we identified a missense mutation in an immunoglobulin (Ig) domain located in the M-line-A-band transition zone of titin, leading to D23186V around the binding site for muscle-specific ring finger protein 1 (MURF1). Analysis of 96 genetically unrelated Japanese patients with familial HCM, who had no previously implicated mutations in known sarcomeric gene candidates, identified two disease-associated mutations in the other Ig domains in proximity to the M-line region of titin. In vitro studies revealed that the mutations found in both medaka fish and in familial HCM increased binding of titin to MURF1 and enhanced titin degradation by ubiquitination. Conclusions: These findings implicate an impaired interaction between titin and MURF1 as a novel mechanism underlying the pathogenesis of HCM.

Section 2: Cardiomyocyte Growth and Regeneration

S2.1 Cardiac hypertrophy in LEOPARD Syndrome is caused by *PTPN11* loss-of-function activity in the developing endocardium

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Mutations in *PTPN11,* encoding the protein tyrosine phosphatase (PTP) SHP2, cause LEOPARD Syndrome (LS), an autosomal dominant disorder with multiple cardiac defects, including hypertrophy. Interestingly, LS mutations are catalytically inactive and behave as loss-of-function. However, how LS mutants affect cardiac development remains unclear. We generated an inducible "knockin" mouse model expressing the LS-associated *Ptpn11* Y279C mutation (iLS/+). When crossed to EIIA deleter-Cre mice, these (now termed) LS/+ mice recapitulated nearly all aspects of the human LS phenotype, including hypertrophy. To determine whether defects in LS/+ mice could be attributed directly to aberrant cardiac developmental effects, we examined hearts from WT, LS/+ and LS/LS embryos at E10.5, E14.5, and E15.5. Both LS/+ and LS/LS developing hearts had significantly diminished trabeculation at E10.5, as well as valvular hyperplasia and ventricular septal defects (VSD) at E14.5, indicative of defective or delayed cardiac development. In addition, LS/LS embryos had persistent VSD at E15.5 and cardiac looping defects that resulted in dextraposition of the aorta, which led to embryonic lethality between E14.5 and E15.5. To determine the lineage-specific effects, we generated neural crest (Wnt1::Cre)-, endothelial (Tie2::Cre)-, and myocardial (Nkx2.5::Cre)- specific LS/+ expressing mouse lines. Surprisingly, we found that only the endocardial-specific LS-expressing mice could completely recapitulate the embryonic cardiac developmental defects observed in the LS/+ phenotype. Moreover, these mice also developed the adult-onset hypertrophy, as observed in adult LS/+ mice. Taken together, our data indicate that the adult-onset cardiac hypertrophy associated with LS is caused by *PTPN11* loss-of-function effects that occur in the developing endocardium**.**

S2.2 Epigenetic regulation of cardiac hypertrophy

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One of the major challenges in managing and treating heart failure patients is to develop disease-modifying drugs that can prevent, reverse, or slow down the disease progression. Upon pathological insults, the heart undergoes remodeling processes, including left ventricular hypertrophy and reprogramming of gene expression. Understanding the mechanisms involved could provide a key to develop interventional therapeutics. Epigenetic modification of chromatin, including histone methylation, regulates gene transcription in response to environmental signals. JMJD2A is a trimethyl-lysine specific histone lysine demethylase. To study the role of JMJD2A, we generated heart specific JMJD2A overexpression and deletion mouse lines. Our studies with these genetically modified mice indicated that JMJD2A is required for pathological cardiac hypertrophy. Furthermore, we show that the demethylase activity of JMJD2A is required for its transcriptional activity. To test whether inhibition of JMJD2A enzymatic activity suppresses hypertrophic response, we identified several small molecule inhibitors of JMJD2A. These small molecule inhibitors of JMJD2A inhibited the phenylephrine-stimulated cardiomyocyte hypertrophy in vitro. Our data suggests that JMJD2A enzymatic activity may act as a hypertrophic determinant and may be an innovative drug target for prevention and treatment of pathological cardiac hypertrophy and heart failure.

S2.3 Regulation of striated muscle gene expression: A role for *Prox1* during cardiac and skeletal muscle development

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Isoform-specific expression of muscle structural genes is a crucial component of the structural, functional and metabolic distinction that exists between the fibre types comprising striated cardiac and skeletal muscle. The homeobox transcription factor *Prox1* has previously been shown to be expressed in slow-twitch skeletal muscle in zebrafish and to be required for normal cardiac muscle development in mice. Using *Nkx2.5-Cre*, *Myf5-Cre* and *Prox1^{flox}* mouse lines to knockout *Prox1* in both striated muscle types, we can identify for the first time a role for *Prox1* in the repression of three key fast-twitch skeletal muscle genes, *Tnnt3*, *Tnni2* and *Myl1*, in both cardiac and slow-twitch skeletal muscle. *Prox1* has an additional role in skeletal muscle in the regulation of myosin heavy chain isoform expression. This implicates *Prox1* in a complex regulatory network that determines fibre type in skeletal muscle, where it functions downstream of *Sox6* and upstream of *Six1*; and characterises a novel role for *Prox1* during heart development. The inappropriate expression of fast-twitch skeletal muscle genes is known to negatively impact cardiac function while some human cardiac and skeletal myopathies have been associated with incorrect isoform expression of structural genes. New opportunities for intervening in the molecular mechanisms that underlie these pathologies will be gained from a better understanding of their developmental regulation.

S2.4 CIP, a novel cardiac nuclear protein regulates cardiac function and remodeling

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Mammalian heart has minimal regenerative capacity. In response to mechanical or pathological stress, the heart undergoes cardiac remodeling. Pressure and volume overload in the heart cause increased size (hypertrophic growth) of cardiomyocytes. Whereas the regulatory pathways that activate cardiac hypertrophy have been well established, the molecular events that inhibit or repress cardiac hypertrophy are less known. Here, we report the identification, characterization and functional examination of CIP, a novel cardiac Isl1-interacting protein. CIP was identified from a bioinformatic search for novel cardiac-expressed genes in mouse embryonic hearts. CIP encodes a nuclear protein without recognizable motifs. Northern blotting, in situ hybridization and reporter gene tracing demonstrated that CIP is highly expressed in cardiomyocytes of developing and adult hearts. Yeast-two-hybrid screening identified Isl1, a LIM/homeodomain transcription factor essential for the specification of cardiac progenitor cells in the second heart field, as a co-factor of CIP. CIP directly interacted with Isl1 and we mapped the domains of these two proteins which mediate their interaction. We show that CIP represses the transcriptional activity of Isl1 in the activation of the MEF2C enhancer. The expression of CIP was dramatically reduced in hypertrophic cardiomyocytes. Most importantly, overexpression of CIP repressed agonist-induced cardiomyocyte hypertrophy. Interestingly, recent work showed that CIP also interacts with Lamin A/C (LMNA), a causative gene mutated in patients with DCM and other disorders. We found that the expression of CIP was markedly regulated in animal models of cardiac diseases, including HCM and DCM. Furthermore, our preliminary data showed that CIP knockout mice display impaired cardiac function. Together, our studies identify CIP a novel regulator of cardiac remodeling and disease.

S2.5 Yap1 regulates cardiomyocyte proliferation through PIK3CB

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Cardiomyocyte loss is a major underlying cause of heart failure. Works focusing on cardiomyocyte proliferation regulation have shown that adult cardiomyocytes do proliferate to a limited extent, but not enough to show any observable clinical benefits. Efforts are needed to deciphering genes capable of driving cardiomyocytes to re-enter cell cycle. The Hippo kinase cascade is an important regulator of organ growth, including heart. A major target of this kinase cascade is YAP1. Our data showed that fetal Yap1 inactivation caused marked, lethal myocardial hypoplasia and decreased cardiomyocyte proliferation, whereas fetal activation of YAP1 stimulated cardiomyocyte proliferation. Remarkably, YAP1 activation was sufficient to stimulate proliferation of postnatal cardiomyocytes, both in culture and in the intact heart. To characterize the mechanism of YAP1 induced cardiomyocyte proliferation, we carried out microarray and Chip sequencing assays. By combining the microarray da ta and the Chip sequencing results, we found several genes that possibly regulated by YAP1.

PIK3CB was validated to be a direct target of Yap1, and a regulatory element residing in the first intron of PIK3CB was characterized. Knocking down of PIK3CB significantly reduced the YAP1 induced cardiomyocyte proliferation, however, TGX221, a specific PIK3CB kinase inhibitor failed to weaken the effects of YAP1 on cardiomyocyte proliferation. Overexpression of either PIK3CB, or PIK3CBK805R, a kinase dead mutation version, was sufficient to drive neonatal cardiomyocytes to re-enter cell cycle in vitro. These studies demonstrate that YAP1 is a crucial regulator of cardiomyocyte proliferation, and YAP1 regulates cardiomyocyte proliferation partially through PIK3CB.

S2.6 A Transitional Extracellular Matrix Instructs Epicardial-mediated Vertebrate Heart Regeneration

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Unlike humans, certain vertebrates including newts and zebrafish possess extraordinary abilities to functionally regenerate lost appendages and injured organs, including cardiac muscle. We aim to discover the underpinning mechanisms that regulate complex tissue rebuilding in these regeneration-competent species, ultimately developing strategies for regenerative wound healing in mammals. Here, we present new evidence that the extracellular matrix (ECM) provides signals to tissues and cells essential for the induction and maintenance of regenerative processes in cardiac muscle. Comprehensive mining of DNA microarrays of regenerating newt and zebrafish hearts with data comparison to the scar-forming response following myocardial infarction in mice and humans provided a signature of conserved regenerative gene activities. Differential expression and Gene Ontology analyses revealed that distinct ECM components and ECM-modifying proteases are among the earliest upregulated and most significantly enriched genes in response to injury in both the newt and zebrafish. In contrast, following mammalian cardiac injury, immune and inflammatory responses are significantly activated instead of ECM genes. Complementary immunohistochemistry studies in the newt demonstrated dynamic spatial and temporal changes in ECM composition between normal and regenerating heart tissues, especially in the epicardium early in the regenerative response. We show in vivo and under defined culture conditions that the proliferative and migratory response of cardiomyocytes is directly linked to distinct matrix remodeling in both the myocardium and epicardium of the regenerating newt heart. Collectively, these results provide a novel understanding of the regenerative process, suggesting that an evolutionarily conserved, regeneration-specific matrix instructs cell activities essential for cardiac muscle regeneration.

2.7 Cardiomyocyte proliferation contributes to post-natal heart growth in humans

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Cardiomyocyte proliferation is an active cellular mechanism during myocardial growth and regeneration in zebrafish, newt, and neonatal mice. We hypothesized that cardiomyocyte proliferation may also contribute to the growth of the human myocardium between birth and adulthood. To test this hypothesis, we analyzed the cellular growth mechanisms in a set of 20 human hearts (age 3 weeks – 20 years) that were procured for transplantation and free from myocardial diseases. Measurements of mitosis, made by automated quantification of phosphorylated histone-3-positive cardiomyocytes, showed that during the first year of life the mean percentage of cardiomyocytes undergoing mitosis was 0.05%, declining to 0.01% at 20 years of age. Cardiomyocyte cytokinesis, visualized by an antibody against MKLP-1 (a component of the centralspindlin complex), was detectable up to 20 years of age, but not later in life. The number of cardiomyocytes, quantified with stereology, increased four-fold between birth and 20 years. The mean cardiomyocyte volume increased concomitantly 12-fold. Relating these mechanistic data to left ventricular mass (determined by echocardiography) showed that cardiomyocyte proliferation contributes 37% to physiologic myocardial growth between birth and 20 years. These findings suggest that altered cardiomyocyte proliferation may be involved in abnormal myocardial growth in congenital heart disease. Our findings also suggest that myocardial regeneration may be present or stimulated in young humans since the underlying mechanism, cardiomyocyte proliferation, is active under the age of 20 years.

2.8 Microarray and Ultrastructure Analyses of a Regenerative Myocardium

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Ciona intestinalis is an invertebrate animal model system that is well characterized and has many advantages for the study of cardiovascular biology. A striking difference between most vertebrates and Ciona is that the Ciona myocardium is capable of regenerating cardiac myocytes throughout its lifespan, which makes the regulatory mechanisms of cardiac myocyte proliferation in Ciona intriguing. In order to identify important regeneration factors in Ciona, microarray analysis was conducted on RNA from adult Ciona hearts with normal or damaged myocardium using custom Affymetrix GeneChips. Hearts were injured via ligation or cryoinjury to stimulate regeneration. After a 24 or 48 hour recovery period, total RNA was isolated from damaged and control hearts. Initial results indicate significant changes in gene expression in hearts damaged by ligation in comparison to cryoinjured or control hearts. Ligation injury shows differential expression of 223 genes as compared to control (fold change >2, $p<0.01$, Student's t-test) with limited false discovery (5.8%). Among these 223 genes, 117 have known orthologs of which 68 were up-regulated and 49 were down-regulated. Ultrastructure analyses of injured myocardium using TEM were conducted in parallel to the microarray study. Preliminary results show changes in the myofibril arrangement and cellular organization in injured hearts. Further studies using immunohistochemistry to identify proliferation and apoptosis in cardiac myocytes of damaged hearts are currently underway. Taken together, these studies will coordinate differences in gene expression to cellular changes in the regenerative myocardium of Ciona, which will help to elucidate the regulatory mechanisms of cardiac myocyte proliferation.

2.9 Deciphering novel molecular mechanisms that facilitate cardiomyocyte de-differentiation and proliferation

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Mammalian cardiomyocytes (CMs) withdraw from the cell cycle shortly after birth and thus lack significant renewal and regenerative capacity. Therefore, inducing CM de-differentiation to enable their cell cycle re-entry could provide the basis for a therapeutic approach for the treatment of cardiac dysfunction following myocardial infarction. In contrast to the adult mammalian heart, the heart of one-day old mice maintains the capacity to completely regenerate after partial resection, by proliferation of mature CMs. The main objective of our work is to decipher the structural and molecular differences between CMs of adult versus neonatal hearts, in attempts to improve the regenerative capacity of the adult mammalian heart. For that, we use multidisciplinary approaches enabling to study the molecular and cellular mechanisms that govern CM proliferation, combining various structural and molecular cell-biology methodologies, using both cell lines and primary cell cultures. Specifically, we characterized CM populations derived from 1d and 7d old mice and determined the expression profile of their cardiogenic genes, as well as their proliferation, differentiation and de-differentiation capacity in vitro. In addition to this characterization, we study how the composition, geometry and forces of the extracellular environment affect the specific features of CMs. Using high-throughput live-cell imaging platforms, we test various signalling pathways for their ability to prime the proliferation, differentiation and de-differentiation of CMs. Preliminary results and future directions of the combined strategy of studying both the traits of CMs together with those of the microenvironment and the possible crosstalk between will be described.

2.10 Tissue specific translational profiling during zebrafish heart regeneration

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Zebrafish have a robust ability to replace lost cardiomyoyctes, and thus provide a unique model to dissect heart regeneration. Genetic lineage-tracing has revealed that regeneration occurs by activating proliferation of pre-existing cardiomyocytes at the injury site. In particular, cardiomyocytes that activate expression of the transcription factor Gata4 are the major contributors of new cardiac muscle. Yet, the molecular mechanisms by which injury activates cardiomyocyte proliferation remain elusive. Here, we have applied the recently developed technology, translational affinity purification (TRAP), to obtain genome-wide profiles of translated mRNAs from different cardiac cell types during regeneration. We created transgenic lines to profile cardiomyocytes, gata4-

positive cardiomyocytes, epicardium, and endocardium, at various times after two different models of cardiac injury. Through translational profiling of cardiomyocytes, we have begun to identify new injury responses by cardiomyocytes that are critical for heart regeneration in zebrafish.

2.11 Rheb is essential for post-natal heart function

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Rheb (Ras homologue enriched in brain) activates mTOR complex 1 (mTORC1) and is an energy sensor. Rheb plays a critical role to keep the balance between cell growth and stress response through regulating protein synthesis and degradation. In this study, we attempted to clarify the role of Rheb in the post-natal heart through studying cardiomyocyte-specific Rheb-deficient mice. Cardiomyocyte-specific Rheb deletion mice were born in Mendelian ratio but started to die 12 days after birth. All of them were lost by 16 days after birth. Echocardiographic analysis revealed that Rheb-deficient mice exhibited cardiac dilatation and reduced contractility 12 days after birth. Electrocardiography recordings showed intermittent complete heart block and sinus arrest in the mutant. These suggest that Rheb-deficient mice died of heart failure and arrhythmia. Rheb-deletion mice also showed reduced heart weight and cardiac fiber size. Downstream of mTORC1, S6 and 4EBP1 were shown to have drama tically reduced phosphorylation levels with enhanced Akt activity. Thus, we concluded that Rheb-mTOR pathway in the heart is essential to regulate heart contractility and electrophysiologic homeostasis at young age.

2.12 *Wdr1* is essential for post-natal and adult heart function

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WDR1, a WD-repeat protein, is highly conserved across all species, from yeast and plants to mammals. it is considered as an important cofactor of cofilin, which serves to accelerate cofilin's activity. AIP1 alone has negligible effects on actin filament dynamics, whereas in the presence of ADF/cofilin, AIP1 enhances filament fragmentation by capping ends of severed filaments. So far, most *in vivo* analyses of AIP1 come from *C. elegans* and few unicellular organisms. unc-78, an AIP1 gene in the C. elegans, is required for organized assembly of sarcomeric actin filaments in the body wall muscle. However, the function of WDR1 in vertebrate muscle has not been reported. Here we generated a WDR1 conditional knockout mouse, *aMHC* promoter drivened cardiac-specific deletion WDR1 in heart caused mice dead from post-natal day 13 to day 24 , accompanied by the increasing of total actin and hypertrophy; to test whether Wdr1 was essential for adult heart, Tamoxifen induced cardiomyocyte-specific deletion of *Wdr1* in adult mice, This ablation resulted in the death within 2 months of Wdr1 deletion. These studies revealed an essential requirement for Wdr1 in post-natal and adult heart functions.

2.13 Preservation of Muscle Force in Mdx3cv Mice Correlates with the Low-level Expression of a Near Full-length Dystrophin Protein

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Complete absence of dystrophin causes Duchenne muscular dystrophy (DMD). Dystrophin restoration at ≥ 20% level reduces muscle pathology and improves muscle force. Levels lower than this are considered therapeutically irrelevant. Interestingly, less than 20% dystrophin expression is seen in some Becker muscular dystrophy (BMD) patients. To understand the role of low-level dystrophin expression, we compared muscle force and pathology in mdx3cv and mdx4cv mice. Dystrophin was eliminated in mdx4cv mice. But mdx3cv mice expressed a near full-length dystrophin protein at ~5% of the normal level. Consistent with previous reports, we found dystrophic skeletal muscle pathology in both strains. Surprisingly, mdx3cv extensor digitorium longus (EDL) muscle showed significantly higher tetanic force and it was also more resistant to eccentric contraction-induced injury. Furthermore, mdx3cv forelimb grip force was stronger. Immunostaining revealed utrophin up-regulat ion and detectable d ystrophin-associated glycoprotein complex assembly on the sarcolemma in both strains. Our results suggest that a sub-therapeutic level expression of a near full-length membrane-bound dystrophin may have contributed to muscle force preservation in mdx3cv mice. This finding may help to explain the benign clinical phenotype in some BMD patients. (Supported by NIH and MDA).

2.14 Conditional Ablation of *Ezh2* in Murine Hearts Reveals Its Essential Roles in Endocardial Cushion Formation, Cardiomyocyte Proliferation and Survival

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Ezh2 is a histone trimethyltransferase that mainly silences genes via catalyzing trimethylation of histone 3 lysine 27 (H3K27Me3). The role of Ezh2 as a regulator of gene silencing and cell proliferation in cancer development has been investigated; however, its function in heart development during embryonic cardiogenesis has not been well studied. We conditionally ablated *Ezh2* in murine heart using the conditional *Ezh2* allele and *Nkx2.5-cre* mice, and identified a wide spectrum of cardiovascular malformations, which led to perinatal death of *Ezh2* mutant mice. In the *Ezh2* mutant heart, the endocardial cushions (ECs) were hypoplastic and displayed impaired endothelial-to-mesenchymal transition (EMT). The mutant heart also exhibited decreased cardiomyocyte proliferation and increased apoptosis. We further identified that the Hey2 gene, which is important for cardiomyocyte proliferation and cardiac morphogenesis, is a downstream target of Ezh2. The regulation of Hey2 expression by Ezh2 may be independent of Notch signaling activity. Our work defines an indispensible role of the chromatin remodeling factor Ezh2 in normal cardiovascular development. *This work was funded by grants HL101336-01(J.W.) from the National Institutes of Health, 09BGIA2050016 (J.W.) and 11POST5680013 (L.C.) from the American Heart Association.*

2.15 QUANTIFYING TISSUE HYPOXIA, HYPOXIC STRESS AND BIOLOGICAL RESPONSES IN THE DEVELOPING EMBRYO

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Reduced oxygen concentrations (tissue hypoxia) and oxygen gradients have been identified and proposed to play a role in heart morphogenesis. Further reductions in oxygen supply, as may occur with vascular insufficiency, high altitudes and anemia, have been linked to congenital heart defects. Critical deficiencies in this field of research include 1) quantifying where and under what conditions tissue hypoxia occurs and 2) linking transcriptional and morphogenic responses to endogenous and evoked hypoxia. For a quantitative approach to tissue hypoxia we have begun to study the ODD-Luc mouse in which Luciferase containing an Oxygen Degradation Domain (ODD) is expressed from the Rosa locus resulting in the accumulation of Luciferase under hypoxic conditions. Luciferase activity is 10-fold higher in the E15.5 embryo as compared to adult tissues consistent with reduced oxygen concentrations. Exposure of E9.5 mice to hypoxic stress (8% O_2 for 4 hrs) increased luciferase activity by 2 fold in the embryos and much less in the mother's tissues. We have previously observed that this level of hypoxic stress, in combination with the iron chelator DMOG, induces a hypoxic transcriptional response in the embryonic (St25) chick heart. Transcriptional profiling in the embryonic mouse heart is in progress. We conclude that ODDLuc provides a quantitative measure of tissue hypoxia in the developing heart. The mouse embryo develops under significant tissue hypoxia and is particularly susceptible to oxygen deprivation early in development.

2.16 An essential role for RNA binding protein Bicc1 in cardiac development and function

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A mutant named *Destro* was recovered from our ongoing mouse ENU mutagenesis and found to have complex congenital cardiovascular defects (CHD) associated with heterotaxy - the randomized patterning of the left-right body axis. Some mutants exhibit complete mirror reversal of heart and visceral organ situs, but such mutants do not have CHD. Mutants also can have cysts associated with the kidney, biliary duct and pancreas. Using whole genome sequencing, we identified the mutation as *Bicc1*$^{c.606+2T>C}$, a splicing mutation causing in-frame deletion of 18 amino acids in the K homology domain involved in RNA recognition. This result is consistent with genome scanning analysis which mapped the mutation to the interval of mouse chromosome 10 containing *Bicc1*. Genotyping analysis showed some homozygous mutants with situs solitus are adult viable, but transthoracic echocardiography revealed cardiac function markedly declined with age. This was associated with dilated cardiomyopathy and marked increase in the expression of heart failure genes such as *osteopontin*. Interestingly, mapping analysis by genome scanning suggested linkage of a modifier locus on chromosome 5

affecting term viability of mutants, an interval containing polycystin-2 and osteopontin. As *Bicc1* is known to regulate polcystin-2 expression via modulation of mir-17 activity, this would suggest *Bicc1* may be required for maintenance of cardiomyocyte homeostasis through microRNA regulation of cardiomyocyte differentiation/function. This may involve miR-539, as consensus binding site for this microRNA is found in both polycystin-2 and osteopontin. Overall, these findings suggest a novel role for Bicc1 in the modulation of microRNAs involved in dilated cardiomyopathy. Supported by U01-HL098180.

2.17 AKAP13 Rho-GEF and PKC-PKD Binding Domains Are Not Required for Development But Are for a Cardiac Functional Response to Chronic β-Adrenergic Stimulation

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Background: A-kinase anchoring proteins (AKAPs) are scaffolding molecules that coordinate and integrate G-protein signaling events to regulate development, physiology, and disease. One family member, AKAP13, integrates G_s, $G_{q/11}$, and $G_{12/13}$ signals through its Rho-guanine nucleotide exchange factor (Rho-GEF) domain and by binding protein kinase A, C (PKC), and D (PKD). AKAP13 appears to be required for development as null mouse embryos die by E10.5 with cardiovascular phenotypes. Additionally, the AKAP13 Rho-GEF and PKC-PKD binding domains mediate cardiomyocyte hypertrophy in cell culture. However, the requirements for the Rho-GEF and PKC-PKD binding domains during development and cardiac hypertrophy are unknown. ***Methodology/Principal Findings:*** To determine whether AKAP13 Rho-GEF activity and AKAP13-bound PKC-PKD are required for development, we generated mice that lacked the Rho-GEF and/or the PKC–PKD binding domains using gene-trap mutational events. Surprisingly, mutant mice were obtained at Mendelian ratios, had normal viability and were fertile. Adult mutant mice also had normal cardiac structure and electrocardiograms. To determine the role of these domains during chronic β-adrenergic-induced cardiac hypertrophy, we stressed mice with isoproterenol. We found that mice lacking the Rho-GEF and PKC-PKD binding domains had the same increase in heart size as control mice. However, these mutant hearts failed to increase cardiac ejection fraction or fractional shortening. ***Conclusions:*** Our results indicate that the AKAP13 Rho-GEF and PKC-PKD binding domains are not required for normal mouse development, cardiac architecture or function, or the structural response to β-adrenergic-induced cardiac hypertrophy. However, these domains are necessary for the cardiac functional response to chronic β-adrenergic stimulation.

2.18 Optical coherence tomography captures rapid hemodynamic responses to acute hypoxia in the cardiovascular system of early embryos

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Background: The trajectory to heart defects may start in tubular and looping heart stages when detailed analysis of form and function is difficult by currently available methods. We used a novel method, Doppler optical coherence tomography (OCT), to follow changes in cardiovascular function in quail embryos during acute hypoxic stress. Chronic fetal hypoxia is a known risk factor for congenital heart diseases (CHDs). Decreased fetal heart rates during maternal obstructive sleep apnea suggest that studying fetal heart responses under acute hypoxia is warranted. Results: We captured responses to hypoxia at the critical looping heart stages. Doppler OCT revealed detailed vitelline arterial pulsed Doppler waveforms. Embryos tolerated 1 hr of hypoxia (5%, 10%, or 15% O_2), but exhibited changes including decreased systolic and increased diastolic duration in 5 min. After 5 min, slower heart rates, arrhythmic events and an increase in retrograde blood flow were observed. These changes suggested slower filling of the heart, which was confirmed by four-dimensional Doppler imaging of the heart itself. Conclusions: Doppler OCT is well suited for rapid noninvasive screening for functional changes in avian embryos under near physiological conditions. Analysis of the accessible vitelline artery sensitively reflected changes in heart function and can be used for rapid screening. Acute hypoxia caused rapid hemodynamic changes in looping hearts and may be a concern for increased CHD risk. (Grant support: RO1HL083048, R01HL095717, T32EB007509, C06RR12463-01)

2.19 The Transcription Factor PPARδ may be involved in Cardiac Morphogenesis

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Deletion of Ppard, encoding the ligand-inducible nuclear receptor peroxisome proliferator-activated receptor δ (PPARδ), in mice is believed to cause mid-gestation death as a result of placental defects, but cardio-specific deletion of the gene with α-MyHC-Cre leads to post-natal cardiomyopathy and death by 4-10 months of age. In addition, PPARδ is ubiquitously expressed, including the embryonic heart, and we have shown that it modulates transcription of the 14-3-3ε protein (Ywhae), which in turn regulates ventricular morphogenesis. Thus, we hypothesized that PPARδ might be involved in heart development. We confirmed that PPARδ is expressed in the heart during development, and showed that the PPARδ-specific agonist L-165,041 upregulates 14-3-3ε levels in P19CL6 cells differentiating toward the cardiomyocyte lineage and primary cardiomyocytes. Administration of 30 mg/kg/day L-165,041 for three days to pregnant females si gnificantly upregulated Ywhae mRNA levels in E12.5 wildtype and Ywhae+/- embryonic hearts. We also found that Ppard-/- mice present significant ventricular dilatation at E9.5, and we are currently deleting Ppard with a cTNT-Cre driver and performing ECHOs in E9.5 Ppard-/- mice to evaluate both umbilical artery flow and cardiac function to better assess the relative role of PPARδ in the placenta and heart. These results indicate that PPARδ modulates cardiac 14-3-3ε, a regulator of ventricular morphogenesis, and suggest that PPARδ may directly control heart development in mice.

2.20 Mechanisms that control the function of the mitochondrial permeability transition pore during cardiac development and myocyte differentiation

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We recently found that changes in mitochondria in the embryonic mouse heart are regulated by the mitochondrial permeability transition pore (mPTP) and closure of the mPTP decreased oxidative stress that facilitates cardiac myocyte differentiation. These experiments suggested that the changes in the expression and/or function of mPTP-regulatory protein, CyP-D, regulate mPTP function and myocyte differentiation in the early heart. To test this hypothesis, early embryonic hearts were harvested from wild-type (WT) and CyP-D null mice for analysis of whole hearts and primary myocyte cultures. Mitochondrial and mPTP function and myocyte differentiation were examined using biochemical assays and fluorescence microscopy. Gene and protein expression and post-translational modification was assessed using RNASeq, immunoblotting (IB), immunofluorescence microscopy (IF) and infection of myocytes with WT and mutated CyP-D expression vectors. Between E9.5 and 11.5, expression of CyP-D and other components of the mPTP generally increased by 20%. IB experiments showed similar small changes in protein expression, while IF of sectioned hearts demonstrated increased CyP-D expression in the less differentiated cells of the ventricular walls compared to the more differentiated trabeculae. Re-expression of WT CyP-D in CyP-D null myocytes blocked the maturation of mitochondrial structure and function and the increased myocyte differentiation we have previously observed in CyP-D null hearts. In contrast, expression of inactive CyP-D had no effect, suggesting that the peptidyl-prolyl isomerase activity of CyP-D is important for these processes. These studies suggest the activity of CyP-D regulates the mPTP and plays a critical role in cardiac myocyte differentiation in the embryonic heart.

2.21 A SENP5-Dependent deSUMOylation Pathway for Dilated Cardiomyopathy

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SUMOylation of proteins regulates diverse cellular processes related to transcription, apoptosis, cell cycle, and protein stability. Sentrin protease 5 (SENP5) is a SUMO2/3 specific deconjugation enzyme and its functional roles in the cardiac pathophysiology remain unknown. Here we show that transgenic mice expressing SENP5 in the heart (SENP5 Tg) develop dilated cardiomyopathy and heart failure that mimic human heart disease with fibrosis, and impairment in myocardial function. Elevated apoptosis and reduced cell proliferation were also

observed in the hearts of these animals. Surprisingly, the hearts from SENP5 Tg mice have markedly reduced mitochondria enzymatic activities due to almost complete loss of mitochondrial cristae, and compromised cardiac energy metabolism. Moreover, the Ca2+ deposition was significantly enhanced in SENP5 Tg mouse hearts. These findings identify that a novel SENP5-mediated deSUMOylation pathway controls mitochondrial function and concomitant pat hological remodeling of the heart. Finally we also observed increased level of SENP5 in human failing hearts. Thus a pharmacologic intervention of SENP5 might be a promising therapeutic approach to prevent dilated cadiomyopathy and heart failure.

2.22 The role of PDGF signaling on zebrafish heart regeneration

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In contrast to mammalian adult hearts, zebrafish hearts regenerate their damaged hearts. To date, cardiac injury methods utilized to study zebrafish heart regeneration include 20% ventricular resection, cryoinjury and genetic ablation. It remains to be determined if zebrafish hearts respond differently to different types of injury. A major difference between cryoinjury and amputation is that dead cells and tissues remain in the heart after cryoinjury, mimicking the pathogenesis after myocardial infarction. It was reported that scar tissue does form after cryoinjury, but it later absorbed and zebrafish hearts still regenerate within 60-120 days post cryoinjury (dpc). However, we observed that 30-50% of zebrafish hearts still have collagen scar by 120dpc. The discrepancy between our data ad the published report might result from initial injury sizes and suggests that a threshold for cryo damage might exist for the regenerative capacity of zebrafish heart. We previously showed that blocking PDGFR signaling decreases epicardial cell proliferation, epithelial-to-mesenchymal transition, and new coronary vessel formation during regeneration after amputation. Using transgenic fish expressing a dominant-negative form of PDGFRβ, we will determine the functions of PDGF signaling in heart regeneration after cryoinjury.

2.23 Role of LEK1 in cardiac disease and function

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In our lab, we have developed global as well as heart-specific CENPF-/- (LEK1) mouse models. Utilizing our heart-specific model, we have found that LEK1 mutation leads to a myriad of problems including: an overall decrease in intercalated disc number, significant fibrosis, erratic ECG patterns, progressive dilated cardiomyopathy, and a 20% chance of sudden death. To date, the molecular mechanism underlying LEK1 involvement in these processes remains unknown. Although LEK1 is a very large and multi-functional protein, one common functional theme has emerged in the literature: the interaction of LEK1 with the microtubule cytoskeleton. Previous studies have identified an important role for LEK1 in microtubule nucleation at the centrosome as well regulation of vesicular transport and cell coupling. Here, using CENPF-/- mouse embryonic fibroblasts, we show that directionally persistent migration as well as overall microtubule network organization is disrupted. Our overall hypothesis is that cytoskeletal defects mediated by LEK1 mutation result in developmental, morphogenic, and functional abnormalities in the heart.

Section 3: Genomics and Gene Regulation

S3.1 Isolation of nuclei from specific cardiac lineages in zebrafish for genome-wide and epigenomic analyses

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To understand the critical gene regulatory interactions that drive atrial versus ventricle identity, we are using genome-wide analysis of differentiating cardiomyocytes to uncover changes in epigenetic and transcriptome profiles. Although advances in sequencing technology have greatly enhanced our ability to characterize genome-wide changes in epigenetic modifications and gene expression, this has been constrained by the technical challenges faced in purifying specific cell lineages in adequate amounts from developing systems. Therefore we are developing a novel approach that will allow us to isolate nuclei of differentiating cardiomyocytes *in-vivo*. We have created transgenic lines in which a biotin ligase recognition peptide (BLRP) is fused to an outer membrane nuclear envelope protein (NEP). Regulated expression of BirA enzyme drives the

in vivo biotinylation of BLRP-NEP, allowing purification of nuclei with streptavidin-conjugated beads. When BirA is ubiquitously expressed, we can successfully elute chromatin threefold higher from transgenic embryos with biotinylated nuclei than transgenic embryos devoid of BirA enzyme. We successfully performed ChIP with multiple histone marks, indicating that the chromatin within these purified nuclei is useful for epigenomic analyses. We are creating double transgenics in which BLRP-NEP is biotinylated in specific heart lineages in order to capture chromatin and nuclear RNA (mRNA and miRNA) from cardiac progenitors, ventricle precursors and atrial precursors. This will allow us to perform genome-wide analysis of epigenetic and transciptome profiles in specific subsets of lineages and tissues during any stage of zebrafish cardiac development. Supported in part by U01HL098179 (NHLBI Bench-to-Bassinet program)

S3.2 TU-tagging in mice defines dynamic gene expression programs in specific cardiovascular or other cell types within intact tissues

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Changes in gene expression programs underlie all aspects of cardiovascular development and disease. Therefore, transcriptional profiling is a powerful and direct approach to study these and other biological processes. Current methods are limited by difficulties isolating specific cell populations from complex tissues, changes in gene expression that occur during sample preparation, and the inability to distinguish dynamic transcriptional changes within a cellular pool of pre-existing RNA. To overcome these limitations in mouse research, we have developed a method called “TU-tagging” that allows for purification of newly transcribed RNA from defined cell types. With TU-tagging, genetic and chemical methods intersect to provide spatiotemporal controlled RNA labeling in vivo. Cre-induced expression of UPRT using a newly-developed transgenic mouse (*CA:loxStoploxUPRT*) directs the incorporation of injected 4-thiouracil (4TU) into nascent RNA, generating a pulse of cell type-specific thiolated RNA. The thio-RNA is readily purified from whole tissue RNA preparations and RNA-seq is used to define “acute” transcriptomes. We validate mouse TU-tagging by using *Tie2:Cre;CA:loxStoploxUPRT* double transgenic mice to purify endothelial RNA from brain or endothelial/endocardial RNA from heart at both embryonic and postnatal stages. In each case, the vast majority of the most enriched transcripts are verified as being expressed in *Tie2:Cre* marked cell lineages. We define a core set of pan-endothelial transcripts and identify groups of endothelial/endocardial lineage transcripts with shared specific expression patterns. TU-tagging provides a novel, sensitive method for characterizing transcriptome dynamics in rare cell types within the intact mouse.

S3.3 Escape of Gene Body DNA Methylation in Cardiac Genes is Cooperative with the Cell Type-specific Expression Patterns

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Mammalian genome is highly methylated in C-G dinucleotides (CpGs), over 60% of which are believed to be methylated. The effect of DNA methylation has been investigated intensively in the promoter regions, but it has still been unknown in non-promoter regions, especially in the cell type-specific effect. Although we previously reported that gene body DNA methylation levels are correlated with the transcription levels and the replication timing in human cell lines (Suzuki et al. Genome Res 2011), function of gene body DNA methylation levels in developing cells is unknown. In this study, we focused on the DNA methylation pattern in cardiomyocytes as the established differentiated cells. We examined genome-wide DNA methylation profiles of cardiomyocytes and non-cardiomyocyte cells from different developmental stages by the deep sequencing technique, and found that the gene body regions of some cardiac genes, including myosin heavy chain (MHC) genes, are less methylated than other genes, where the DNA methylation levels were dramatically decreased in the latter half of pregnancy. To evaluate the effect of gene body DNA methylation, we performed luciferase reporter assay by the reconstruction using the SssI-methylated and unmethylated gene body fragments, and confirmed that the gene body DNA methylation is repressive in the gene expression, mostly at the transcriptional level. Collectively, the gene body DNA methylation levels in some cardiac genes were developmentally regulated and possibly enhance the transcription of cardiac genes.

S3.4 The novel mechanism of histone-chromatin regulation for cardiomyocytes regeneration

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Fishes and amphibians have high capacities for heart regeneration even in the adult, whereas we mammalians lose such regeneration capacities. Understanding the mechanism of heart regeneration should provide the most important cues on the therapies for human heart failure. We found that SWI/SNF-BAF type cardiac chromatin remodeling factors and histone regulators were strongly up-regulated within 12 hours after resection of ventricle both in neonatal mice and axolotl, and these expression was maintained for one week during regeneration. To address whether these factors function as the early response factors in mammalian heart regeneration, we constructed BAF-overexpression (BAF-TG) mice, which showed stable expression of BAFs even in the adult heart. Interestingly, BAF-TG prevented fibrosis post myocardial infarction and regenerated their injured parts with the proliferation of cardiomyocytes. In vivo ChIP analyses revealed that the SWI/SNF core factor, Brg1, directly regulated several cardiac fetal genes' promoters such as Nppa, Tnnt2, Myl7 only at the embryonic stages. Surprisingly, in the BAF-TG, major cardiac fetal genes' promoters were still opened in the adult state. And si-treatment of polycomb or repress-type chromatin factor s increased cell proliferation in vitro. These data demonstrate that histone-chromatin conformation is changed depending on the developmental stages on several cardiac genes relating with the regeneration capacity.

S3.5 Epigenetic regionalization of the developing mouse heart

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A multitude of differences in gene expression have been described and correlated with cardiac chamber identity, structure, function and disease. Nonetheless, it is not fully resolved how these transcriptional spatial asymmetries, evident at even the very early stages of morphogenesis, are established and maintained. An intriguing hypothesis is that these asymmetries are contributed to by differences in chromatin accessibility, which potentiate or exclude expression of specific genes. By performing transcriptional profiling by RNA-seq on isolated left and right ventricles of embryonic mice and correlating the findings with ventricular ChIP-seq data for H3K27me3, a mark of closed chromatin associated with gene repression, and H3K4me1, a histone modification associated with open chromatin and active genes, we observe a significant subset of chamber-specific cardiac genes, including Tbx5, that are selectively marked by both H3K4me1 and H3K27me3 in opposite chambers. In the left ventricle, these genes are functionally linked to roles in energy metabolism, whereas in the right ventricle, coordinately marked genes are associated with developmental roles. We demonstrate that Tbx5 enhancers have the potential of driving expression in broad cardiac domains, and that the restricted Tbx5 expression is mainly due to regionalization of epigenetic marks. Furthermore, this spatially regionalized pattern of gene repression is redeployed to limit Tbx5 expression to forelimb and not hindlimb, highlighting a general chromatin-based mechanism to affect spatially-restricted expression. Beyond our general understanding of how the developing heart is patterned, these findings have implications for efforts at directed differentiation of stem cells into cardiomyocytes for therapeutic uses.

S3.6 *Drosophila* microRNA-1 Genetically Interacts with *little imaginal discs*, a histone demethylase.

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The expression pattern and function for the microRNA *miR-1* is conserved in both invertebrates and vertebrates, suggesting that it modulates some of the most fundamental processes within cardiac and skeletal muscle, including Notch signaling. We performed a forward genetic screen in *Drosophila* that identified *little imaginal discs* (*lid*), a chromatin-remodeling enzyme, as a genetic partner of *dmiR-1*. Lid demethylates histone H3 lysine 4 (H3K4), thereby epigenetically decreasing the activity of selected promotors. Lid forms a complex with Notch pathway regulatory proteins, and loss of lid activity results in de-repression of Notch downstream targets. We have found that in flies, heterozygosity of *lid* in the context of *dmiR-1* overexpression resulted in exacerbation of the *dmiR-1* overexpression phenotype, consistent with de-repression of Notch signaling. Furthermore, we discovered that use of a balancer chromosome containing a mutation in the Notch ligand *Serrate*, unmasked an indirect flight muscle phenotype in $lid^{(-/-)}$ flies that was modified upon manipulation of *dmiR-1* levels. These

results imply that *dmiR-1* may selectively modulate the epigenetic modification of histones at Notch-responsive loci during fly heart and muscle development.

3.7 Mutual Regulation of Nkx2.5 and Fibulin-1 in the SHF Regulatory Network

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Approximately 12,000 children are born yearly with malformations of the aorta and/or pulmonary artery resulting from abnormal outflow tract (OFT) development. The cardiac OFT arise from progenitor cells of the second heart field (SHF) and animal studies have shown that this process is subject to the influence of several transcription factor, growth factor and extracellular matrix (ECM) genes. It is therefore likely that the combinatorial effects of multiple gene mutations underlie genetically complex OFT congenital heart defects (CHD) entities in humans. As part of an effort to determine functional linkages between known OFT developmental regulators, we have found evidence for mutual regulation of the cardiac transcription factor Nkx2.5 and the ECM protein fibulin-1 (Fbln1), both of which are required for normal SHF and OFT development in mice. Specifically we have found that Fbln1 expression levels are decreased in SHF regions in Nkx2.5 null mice, and that Nkx2.5 appears to regulate Fbln1 expression in the SHF via binding to an evolutionarily conserved NKE binding site in promoter proximal regions. Conversely, Fbln1 expression reciprocally regulates Nkx2.5, as Nkx2.5 expression is decreased in SHF regions of Fbln1 null mice in association with increased expression of a potential direct repressor of Nkx2.5, the growth factor activated transcription factor Egr1. These findings support the hypothesis that Nkx2.5 and Fbln1 are key components of a growth factor-mediated homeostatic mechanism regulating SHF proliferation and OFT development.

3.8 FOG-2 Mediated Recruitment of the NuRD Complex Regulates Cardiomyocyte Proliferation during Heart Development

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FOG-2 is a multi-zinc finger protein that binds GATA4 and modulates GATA4-mediated transcriptional control of target genes during heart development. Our previous work has demonstrated that the Nucleosome Remodeling and Deacetylase (NuRD) complex physically interacts with FOG-2 and is necessary for FOG-2 mediated repression of GATA4 activity in vitro. In this report, we describe the generation and characterization of mice homozygous for a mutation that disrupts FOG-2/NuRD complex interaction (FOG-2^{R3K5A}). These mice exhibit a perinatal lethality and have multiple cardiac malformations, including a ventricular septal defect and a thin ventricular myocardium. To investigate the mechanism underlying the thin ventricular walls, we measured the rate of cardiomyocyte apoptosis and proliferation in wild-type and FOG-2^{R3K5A} developing hearts. We found no significant difference in the rate of cardiomyocyte apoptosis between wild-type and FOG-2^{R3K5A} mice. However, cardiomyocyte proliferation was reduced by 29.8% in FOG-2^{R3K5A} mice. Interestingly, we found by gene expression analysis that the cell cycle inhibitor p21 is up-regulated 1.7-fold in FOG-2^{R3K5A} hearts. Further, we demonstrate that FOG-2 can directly repress the activity of the p21 gene promoter using an in vitro transient transfection assay. In addition, chromatin immunoprecipitation (ChIP) reveals that the NuRD complex is bound to the p21 promoter in wild-type embryonic day 14.5 hearts but is diminished at this promoter in mutant hearts. Taken together, our results suggest the FOG-2/NuRD interaction is required for cardiomyocyte proliferation by specifically down-regulating expression of the cell cycle inhibitor p21 during heart development.

3.9 A strict lineage boundary between the first and second heart fields is defined by the contribution of the Tbx5 lineage.

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The theory that the heart arises from two distinct heart fields suggests important clues as to how the heart is patterned. Determining if contributions of distinct first heart field (FHF) and second heart field (SHF) cell populations to the fully formed heart exist is central to understanding heart development and the etiology of congenital heart defects (CHDs). Haploinsufficiency of the transcription factor TBX5 causes Holt-Oram Syndrome, which includes CHDs. Tbx5 expression outlines a region of the heart that is presumed to correspond

to the FHF, and Tbx5 is required in the posterior half of the heart. However, it is not known what structures within the heart the Tbx5 lineage gives rise to, or how these relate to the importance of Tbx5 in CHDs. Using an inducible Cre recombinase inserted at the Tbx5 locus, and various SHF genetic markers, we have mapped the Tbx5-expressing lineage and its intersection with the SHF, to discern field allocation in cardiac morphogenesis. We fi nd that from the earliest stages of Tbx5 expression, prior to formation of the linear heart tube, the Tbx5 lineage contributes to the posterior segments of the heart, ending at a sharp boundary at the interventricular septum, with little overlap with the SHF. We propose that the earliest Tbx5 expressing cells define the FHF lineage, and that a strict lineage boundary exists between the FHF and SHF prior to morphogenic distinctions between their descendents. Understanding the contribution of Tbx5 to the FHF will contribute to our knowledge of heart development and CHDs.

3.10 Tbx5-Hedgehog pathway is required in second heart field cardiac progenitors for atrial septation

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The developmental mechanisms underlying human Congenital Heart Disease (CHD) are poorly established. Atrial Septal Defects (ASDs), a common form of CHD, can result from haploinsufficiency of cardiogenic transcription factors including Tbx5. We demonstrate that Tbx5 is required outside the heart in second heart field (SHF) cardiac progenitors for atrial septation in mice. Conditional Tbx5 haploinsufficiency in the SHF, but not in the myocardium or endocardium, caused ASDs. Tbx5 SHF knockout embryos lacked atrial septum progenitors. We identified SHF Tbx5- responsive cell cycle progression genes, including cdk6 as a direct target, and Tbx5 mutant SHF progenitors demonstrated a mitotic defect. Genetic and molecular evidence including the rescue of atrial septation in Tbx5 mutant embryos by constitutive Hh-signaling placed Tbx5 upstream or parallel to Hh signaling in cardiac progenitors. We identified Gas1, a Hh-pathway member upstream of Smo, as a direct target of Tbx5. We also found that Osr1, required for atrial septaion in mice, is expressed in a Tbx5-dependent manner. We further identified Osr1 as a direct target of Tbx5 required for atrial septation. These results describe a SHF Tbx5-Hh-signaling network and independent Tbx5-Osr1 pathway required for atrial septation. Ongoing work is aimed at identifying molecular links between these pathways in atrial septation. A paradigm defining molecular requirements downstream of Tbx5 in cardiac progenitors as organizers of cardiac septum morphogenesis has implications for the ontogeny of CHD.

3.11 The Six/Eya transcription complex in cardiac outflow tract development and disease

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<u>*Background*</u>: The *Six*-family genes (*Six1-6*) encode the homeodomain-containing transcription factors. They physically interact with the Eya-family cofactors (Eya1-4) to form the canonical Six-Eya transcription complex. We recently reported that *Six1* and *Eya1* are critical for cardiac outflow tract development since compound mouse mutants exhibit severe outflow tract septation and alignment defects. It is unknown, however, whether any other members of the *Six*- and *Eya*-family genes are also involved in cardiovascular development. <u>*Results*</u>: *Six2* is closely related to *Six1* with over 95% sequence homology. To examine whether *Six2* is involved in the cardiac outflow tract development, we examine its expression pattern, genetic lineages, and its functions in mouse mutants. Our findings demonstrate that *Six2* is expressed in a subset of the second heart field progenitors, which primarily contribute to the pulmonary outflow trunk and the right ventricle. Compound *Six2;Six1* and *Six2;Eya1* mutants have a spectrum of cardiac outflow tract defects. *Six2* expression is significantly down regulated in the *Shh* mutant, which exhibits severe pulmonary trunk agenesis phenotype. We find that *Six2*$^+$ lineages are significantly diminished in *Shh* mutants, suggesting that *Shh* signaling regulates the behavior of the *Six2*$^+$ progenitors during pulmonary trunk and right ventricle formation. <u>*Conclusions*</u>: *Six2* marks a critical subpopulation of the second heart field progenitors that give rise to primarily pulmonary but not aortic outflow trunk, and the *Shh-Six2* genetic pathway is essential for pulmonary trunk and right ventricle formation. In addition, we will also present our preliminary targeted genomic screening of DiGeorge patients as we have identified putative mutations in the *Six*- and *Eya*-family genes. These new finding suggests, for the first time, that *Six*- and *Eya*-family genes are directly linked pathogenesis of human congenital heart defects.

3.12 PRC2 Regulates Heart Development and Adult Heart Energy Homeostasis.

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Epigenetic marks are crucial for organogenesis, but their role in heart development and function is poorly understood. Polycomb Repressive Complex 2 (PRC2) trimethylates histone H3 at lysine 27, establishing H3K27me3 repressive epigenetic marks that promote tissue-specific differentiation by silencing ectopic gene programs. Firstly we studied the function of PRC2 in murine heart development using a tissue-restricted conditional inactivation strategy. Inactivation of the PRC2 subunit *Ezh2* by Nkx2-5^{Cre} (Ezh2^{NK}) caused lethal congenital heart malformations, namely compact myocardial hypoplasia, hypertrabeculation, and ventricular septal defect. Candidate and genome-wide RNA expression profiling and chromatin immunoprecipitation analyses of Ezh2^{NK} heart identified genes directly repressed by EZH2. Among these were the potent cell cycle inhibitors *Ink4a/b*, whose upregulation was associated with decreased cardiomyocyte proliferation in Ezh2^{NK}. EZH2-repressed genes were enriched for transcriptional regulators of non-cardiomyocyte expression programs, such as *Pax6*, *Isl1*, *Six1*. EZH2 was also required for proper spatiotemporal regulation of cardiac gene expression, such as *Hcn4*, *Mlc2a*, and *Bmp10*. Moreover, the cardiomyocytes restriced ablation of another PRC2 key component Eed at the neonatal stage caused dilated cardiomyopathy. Further study showed the mutant cardiomyocytes were defect in energy metabolism by increases in both glycolysis and mitochondria respiration, however without change in total ATP production. Glucokinase (*GcK*), a kinase for phosphorylation of Glucose to phosphate-6-glucose in the first step of glycolysis, was largely upregulated in Eed mutant, at least partially contributing to the enhanced glycolysis. Taken together, our study reveals a previously undescribed role of PRC2 in regulating heart formation and energy metabolic homeostasis.

3.13 Alternative splicing regulation during cardiac differentiation

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Alternative Splicing (AS) is a post-transcriptional process affecting ~95% of human genes and one of the major reason for proteome diversity. AS events are strictly regulated during heart development and embryonic stem cell differentiation into cardiomyocytes such that fetal isoforms are expressed only in embryonic but not in adult stages. Such regulation is essential to accommodate the functional differences between embryonic and adult heart. Even though AS is important for appropriate and specific isoforms of cardiac gene expression during development, the signals that regulate these transitions are largely unknown. Here, by using differentiation of H9c2 rat embryonic heart cells into cardiomyocytes, we show that protein kinase C (PKC) activity is critical during cardiac development, and it regulates fetal AS events in embryonic hearts and also during cardiomyocyte differentiation via tight control of CUG-binding protein 1 (CUGBP1). Funding**:** AHA SDG-093022N, March of Dimes Basil O'Connor starter scholar grant (#5 FY12-21) and UTMB start up funds to M.K-M

3.14 MMAPPR: Mutation Mapping Analysis Pipeline for Pooled RNA-seq

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Forward genetic-screens in zebrafish are a vital tool for identifying novel genes essential for cardiovascular development or disease processes. However, one drawback of these screens is the labor-intensive and sometimes inconclusive process of mapping the causative mutation. Recent advances in high-throughput DNA-sequencing technologies have made mapping mutations much more rapid in human populations. However, they have proven less effective in zebrafish because of its limited gene annotation, high polymorphism rate and the costs associated with performing these analyses on a large number of individuals. However, RNA-seq is commonly used on pools of zebrafish and shows strong potential for mapping recessive mutations. We propose an analysis pipeline for mapping mutations using RNA-seq, called MMAPPR (Mutation Mapping Analysis Pipeline for Pooled RNA-seq) to identify the causative mutation underlying an observed phenotype. MMAPPR (1) uses pooled samples without any knowledge of parental or individual genotypes, (2) accounts for the considerable amount of noise in RNA-seq datasets and (3) simultaneously identifies the region where the mutation lies and generate a list of putative coding region mutations in the linked segment that affect protein structure. We have validated our method on two known mutant lines (Nkx2.5 and Tbx1). We have also successfully mapped two novel cardiovascular mutants and are currently sequencing the identified candidate

genes. In addition to zebrafish, MMAPPR can be directly applied to other model organisms, such as *D. melanogaster* and *C. elegans*, where both forward genetic screens and pooled RNA-seq experiments are common. Supported by the NHLBI Bench-to-Bassinet Consortium Grant (U01HL0981)

3.15 Capture and analysis of cardiac lineage-specific gene expression profiles

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While several critical components of cardiovascular development have been elucidated, the gene expression profile and signaling mechanisms that govern these processes is largely undetermined. Temporal and lineage-specific regulation of gene expression occurs throughout development and in response to distinct specific cell signaling events; however, current technology is constrained by the inability to effectively collect purified lineages at important developmental timepoints. To circumvent this problem we have developed transgenic zebrafish lines in which a biotin ligase recognition peptide (BLRP) is fused to a ribosomal protein (Rpl), and other lines in which lineage-specific expression of biotin ligase (BirA) *in vitro* biotinylates BLRP-Rpl. This BLRP-Rpl-BirA technology allows us to capture polysomes from specific cell lineages out of whole embryo lysates. With the lineage-specific polysomes in hand, we can then determine gene expression profiles from distinct tissues/cell lineages at specific developmental timepoints. Using a cardiac lineage specific (cmlc2/myl7) promoter to drive BirA expression, we have captured BLRP-biotin-tagged polysomes from developing cardiac lineages, and performed RNA-Seq and RT-PCR analysis. These gene expression profiles are being used to define cardiac-enriched transcripts. In addition, we are generating transgenic fish lines that specifically drive expression of BirA and BLRP-Rpl biotinylation in several specific cardiac lineages and in response to distinct cell signaling pathways. This powerful method will allow us to obtain genome-wide expression profiles within cardiac lineage at specific timepoints that are critical for heart development. Supported in part by AHA 09POST2260423 and NHLBI 1F32HL114181 postdoctoral fellowships, and U01HL098179 (NHLBI Bench-to-Bassinet program).

3.16 Identification of Intronic Sequence Responsible for Transcriptional Repression of etv2 in Myocardial Progenitors in the Zebrafish

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Gene regulatory networks governing the separation and maintanance of endothelial, endocardial and myocardial lineages from mesoderm remain poorly defined. During embryonic development of vertebrates, Etv2, an ETS family related transcription factor, is known to be indispensable for the initial specification and differentiation of endothelial cell lineages. Recently, FoxC1 factors were found to directly transactivate etv2 in zebrafish. Nevertheless, the transcriptional regulation of etv2 expression remains poorly understood. We applied reporter transgenics, co-expression analyses by fluorescent double in-situ-hybridisation, and morpholino-oligonucleotide mediated knockdown techniques to examine the cis-regulatory input of key transcription factors (TFs) into the etv2 gene locus in zebrafish. We found that reporter transgenes comprising regulatory regions upstream and downstream flanking the etv2 transcription start site robustly recapitulated endogenous etv2 expression in hematopoietic, endothelial and endocardial progenitors. Surprisingly, omission of intronic sequence resulted in ectopic activation of reporter transgene expression specifically in myocardial precursor cells. During heart cone stages (19-21 hpf), these cells co-expressed nkx2.5 and gata5 TFs that are fundamental to heart development. Co-expression with gata5 was seen already at 12 hpf, but not with nkx2.5. Knockdown of nkx2.5 activity enhanced expression of both intron-less and specific intron-harboring transgenes. In contrast, gata5 knockdown decreased expression of these transgenes. Further TFs are currently being investigated and presented at the conference. These studies suggest that we have identified an intronic cis-regulatory element that is responsible in part for repression of the etv2 gene in myocardial progenitors. Our data also indicate a novel genetic mechanism linking endothelial/endocardial and myocardial cell lineages.

3.17 Regulation of Heart Development by miR-17-92

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Germline deletion of the microRNA (miRNA) cluster miR-17-92 in mouse models has previously been shown to

result in neonatal lethality due to impaired lung and B cell development, as well as ventricular septal defects. To determine the roles of miR-17-92 exclusively in heart development, we used cardiac-specific Cre lines with both conditional over-expression and knock-out alleles. Here we show that both over-expression and deletion of miR-17-92 in the cardiac lineage lead to ventricular septal defects and other cardiomyopathies. We are currently analyzing the potential for the miR-17-92 cluster to regulate mRNA expression genome-wide by performing RNA-Immunoprecipitation microarrays ("RIP-chip") of Argonaut 2-bound RNA purified from mutant and control tissues. This method is useful to purify target mRNAs specifically bound to the miRNA-RISC (Ago2) machinery. Analysis of data from the RIP-chip has revealed several candidate target genes that overlap with miRNA target predictions. We are currently validating several of these predicted targets and determining how they interact with miR-17-92. These include several members of the Wnt signaling pathway. Accordingly, when miR-17-92 is conditionally knocked out, we observe decreased active beta-catenin.

3.18 Discovering small molecules that promote cardiomyocyte generation by modulating Wnt signaling

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We have developed a robust *in vivo* small molecule screen that modulates heart size and cardiomyocyte generation in zebrafish. Three structurally-related compounds (Cardionogen-1 to -3) identified from our screen enlarge the size of the developing heart via myocardial hyperplasia. Increased cardiomyocyte number in Cardionogen-treated embryos is due to expansion of cardiac progenitor cells. In zebrafish embryos and murine embryonic stem (ES) cells, Cardionogen treatment promotes cardiogenesis during and after gastrulation, whereas inhibits heart formation before gastrulation. Cardionogen-induced effects can be antagonized by increasing Wnt/β-catenin signaling activity. We demonstrate that Cardionogen inhibits Wnt/β-catenin-dependent transcription in murine ES cells and zebrafish embryos. Cardionogen can rescue Wnt8-induced cardiomyocyte deficiency and heart-specific phenotypes during development. These findings demonstrate that *in vivo* small molecule screens targeted on heart size can discover compounds with cardiomyogenic effects and identify underlying target pathways. *The paper has been published in *Chemistry & Biology.* 2011 Dec 23;18(12):16

3.19 ETS2 and Mesp1 transdifferentiate human dermal fibroblasts into cardiac progenitors

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Heart failure is one of the leading causes of death in industrialized countries. Due to the shortage of available donors for transplants, alternative therapies are being developed which are based on usage of embryonic stem (ES) and induced pluripotency (IPS) cells. Usage of IPS cells in cardiac cell replacement therapy is advantageous because they circumvent inmuno-rejection problems, since they can be produce directly from the patient's own cells. Unfortunately, current techniques of IPS cell production, still lead to low yields of full undifferentiated cells and even lower yields in sequential differentiation procedures. Our study centers on the design of an effective manner to convert Normal Human Dermal Fibroblasts into cardiac progenitors, through the usage of transcription factors v-ets erythroblastosis virus E26 oncogene homolog 2 (ETS2) and mesoderm posterior 1 homolog (Mesp1). The first, ETS2 being a known oncogene, which potentially initiates a proliferative activity by c-Myc control and the second MesP1 an early mesoderm marker implicated in cardiac formation. From the developmental perspective, ETS2/Mesp1 treated cells have significant cardiomyocyte potential. They express an cardiac progenitor program represented by NKX2.5, MEF2C TBX5, GATA4 and ISL1, contractility and electrical coupling represented by MHC3, MLC2, TNT, CX43, CX45, as well as cell surface mesoderm markers Flk1/KDR and PDGFRa required in an obligatory fashion during cardiomyocyte development.

3.20 Cell surface chemoproteomics for capturing states of cardiac differentiation from pluripotent stem cells

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The ability to transplant cardiomyocytes to effect myocardial repair and remuscularization is a major unfulfilled goal of regenerative medicine. To address this, the current study aims to develop a non-transgene-based method to isolate transplantable numbers (tens of millions) of purified, functionally defined cardiomyocytes using immunophenotyping, a strategy well-defined for isolating therapeutically viable hematopoietic stem cells. To identify surface marker panels unique to cardiomyogenic subtypes (e.g. atrial, ventricular), we utilize a state-of-the-art quantitative chemoproteomic approach that does not rely on the availability or specificity of antibodies – high mass accuracy mass spectrometry (MS) coupled with extracellular protein domain capturing – a strategy that enables epitope discovery and quantitation. This strategy is applied to a well-defined mouse embryoid body-based model of cardiomyogenesis to reveal cell surface proteins that quantitatively change during mesoderm commitment and cardiac differentiation. Subsequently, antibody-based cell sorting is used to isolate cardiomyocyte subtypes based on surface epitope expression, followed by molecular, electrophysiological, and functional assessment. To date, this strategy has identified >600 cell surface proteins among the pluripotent and cardiomyocyte populations studied. From these, >30 proteins are signficantly enriched in the cardiomyocyte population. Of these, several candidate proteins were chosen for further analysis by flow cytometry, immunoblotting, RT-PCR, and whole mount immunostaining to determine when and where these markers appear during various stages of differentiation and *in vivo* development. In summary, these results indicate that cell surface proteins display distinct expression patterns during cardiomyogenesis that may enable the sorting of distinct subpopulations of cardiomyocytes for basic science studies and therapeutic applications.

3.21 Gata5 efficiently directs formation of cardiac progenitors with atrial-like potential from murine embryonic stem cells

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Many of the transcription factors controlling cardiogenesis are known and comprise a molecular network essential for normal heart growth, morphogenesis, and function. However, there is less known about the mechanisms that regulate the development of specific cardiac lineages from embryonic stem cells. The transcription factors GATA4/5/6 have been shown to function redundantly in the regulation of cardiac development. In vivo studies suggest that Gata5 may control a genetic program involved in the development and specification of cardiomyocyte fate, including septal development and valvulogenesis. We have developed a system for conditional expression of Gata5 in murine embryonic stem cells (mES), and tested whether Gata5 can direct specification of cardiomyocyte fate, evaluated by qPCR, immunohistochemisty, flow cytometry, and electrophysiology. We show that induction of Gata5 expression at day 4 of embryoid body (EB) development in serum-free conditions results in derivatives that demonstrate extensive contractility. Typically, at least 50% of the cells within Gata5-induced EB derivatives express cardiac troponin T (cTnt) by day 12. A significant proportion of these cells represent immature cardiac precursors as demonstrated by co-expression of cTnt and smooth muscle actin alpha. Additionally, expression of Gata5 within this defined window directs development of atrial-like progenitors as identified by their protein and electrophysiologic profile. These cells express the atrial isoform of myosin regulatory light chain 2, show heterogeneous action potential morphology, and display responsiveness to adrenergic and muscarinic stimulation. These findings suggest that Gata5 functions downstream of known procardiac signaling programs to direct specification of progenitors with atrial-like potential from mES.

3.22 Salls Promote Cardiac Progenitor Differentiation to Specify Cardiac Cell Lineages

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The fundamental approaches what we should progress in mammal cardiac program research will be to identify

defined factors for induced cardiac stem cells/progenitor cells (iCPCs), and to generate the methods for induction of cardiac cells with high efficiency through their progenitor cell-fate in vivo/in vitro systems. The knowledge gained from the developmental studies led to the recent breakthrough discoveries of 3 defined factors, whose co-overexpression is sufficient to “direct (re)-program” non-cardiac cells to convert into cardiomyocytes. Based on these findings, we have identified Sall genes as the new candidate markers to CPCs from 15 genes that were highly enriched in Islet/Flk1(+)-CPCs in mice embryos. Sall-EGFP knock-in ES cells and sorted Sall(+) cells from mouse embryos significantly differentiated into ventricular-like cardiomyocytes, conduction cells including SAN, smooth muscle and endothelial cells, indicating Sall(+) cells position upstream of Islet1/Nkx2-5-WT1-CPCs. Surprisingly, mostly Sall-EGFP(+) colonies differentiated into beating cardiomyocyte (80%) with cardiac Troponin (>90%), whereas EGFP(-) cells differentiated into cardiomyocytes with low efficiency (<20%). These results suggest that these genes have potential to help human cardiac stem cell research towards the clinical application. In this meeting, we will show that these candidate genes play as defined factors converting embryonic mesoderm or ES cells to induced cardiac progenitor cells (iCPCs) by co-overexpression experiment.

3.23 PDGFR alpha Regulates Flk-1 via Activin/Nodal and BMP Signaling

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In-vitro ES cell systems are becoming widespread as a tool for investigating lineage differentiation. To establish a system for analyzing cardiac mesoderm differentiation induction into discrete cardiomyocyte lineages, we have evaluated Bry, Flk1 and PDGFRα as potential cardiogenic markers following induction by Activin/Nodal and BMP. We found that Bry+/PDGFRα+/Flk-1+ and Bry+/PDGFRα+/Flk-1- populations could both be induced to contractile cardiomyocytes by beating cellular phenotype and expression of Nkx2-5 and cardiac specific MHC. This surprising observation suggested that Flk-1 expression is not informative in the mesoderm to cardiac mesoderm decision. Bry+/PDGFRα-/Flk-1+ population did not generate cardiomyocytes, and instead expressed Gata1 and vWF, consistent with the known role for Flk-1 in blood and endothelial lineages. Our observations suggested that PDGFRα may instead be a useful marker for cardiac mesoderm. By time course study , we observed that the Bry+/PDGFRα+/Flk-1- population generated a Bry+/PDGFRα+/Flk1+ population by Activin A and BMP4 signaling together but not by either Activin A or BMP4 alone. The Bry+/PDGFRα+/Flk1+ population then bifurcated to generate Bry+/PDGFRα-/Flk1+ vascular and endothelial progenitors. These observations suggested the specificity of PDGFR signaling for cardiac differentiation, and Flk-1 action for vascular and endothelium differentiation. When PDGFR signaling was blocked with small molecules early, we observed that the Bry+/PDGFRα+/Flk1+ and Bry+/PDGFRα-/Flk1+ populations were reduced in a dose-dependent manner. We are investigating the working hypothesis that Bry+/PDGFRα+/Flk-1- mesoderm differentiates into Bry+/PDGFRα+/Flk1+ for cardiac mesoderm, and Bry+/PDGFRα-/Flk1+ for vascular progenitors.

3.24 CENP-F deficiency in mouse embryonic stem cells alters BMP expression patterns and impairs cardiomyocyte differentiation.

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CENP-F is a microtubule binding protein that is highly expressed in the developing heart. Previous studies by our lab have shown a temporal correlation between CENP-F protein expression and BrdU incorporation in normal cardiomyocytes in the first week after birth; downregulation of CENP-F in the neonatal mouse heart is concurrent with cessation of cardiomyocyte mitosis. Studies from our lab and others also suggest an early role for CENP-F in the process of cardiomyocyte differentiation. We have created embryonic stem (ES) cell lines from a previously generated CENP-F flox/flox mouse model with a tamoxifen driven cre-recombinase. Addition of low dose tamoxifen to these cells results in deletion of CENP-F. In differentiation assays, tamoxifen treated cells exhibit impaired differentiation into the cardiomyocyte and endothelial cell lineages, while expressing high levels of endodermal and smooth muscle markers. Quantitative real time PCR confirms low levels of cardiomyocyte markers and altered patterns of BMP 2 and 4 expression in treated vs. untreated cells. We are in the process of expanding these analyses to other cell lineages to better characterize the effects of loss of CENP-F in this model of differentiation.

3.25 Combinatorial Regulation of Cardiac Gene Expression by ETS and Mesp1 transcription factors

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Mesp1 plays a key role in cardiac lineage commitment contributing to the development of both the first and second heart fields. It is a bHLH transcription factor that binds to the conserved E-box sequences in DNA. Current knowledge of the downstream genes and protein partners of Mesp1 is limited. Among potential Mesp1 partners are the ETS family transcription factors which contain conserved DNA-binding ETS domains and are also essential for cardiac development. Thus, transcripts of several ETS factors are abundant in the early embryonic heart, whereas ETS2-knockout ES cells fail to beat and do not express cardiac markers. We have started the Chip-seq analyses of the mouse embryoid bodies using antibodies to endogenous Mesp1 and ETS family proteins to determine global distribution of their binding elements. Gene Ontology analysis relates an abundance of determined closely spaced E-box/ETS binding sites to the upstream sequences of the genes involved in cardiac development. Initial tests of such elements by the Luciferase assay have shown that pair-wise binding of Mesp1 and several ETS factors to the upstream sequence of the early cardiac gene Nkx2-5 results in a synergistic transcriptional activation. Co-immunoprecipitation has shown physical association between several ETS transcription factors and Mesp1. Thus, our studies indicate that physical and functional interactions between Mesp1 and ETS transcription factors may play important roles in cardiac development.

3.26 Dissecting Tbx5 function: roles for the novel Tbx5b paralog in cardiogenesis

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Congenital Heart Disease (CHD) is the most frequent birth defect, and mutations in transcription factors, including T-box proteins, are causative in many of these CHDs. Mutations in one family member, TBX5, results in the autosomal dominant condition Holt-Oram Syndrome (HOS), characterized by heart malformations and limb deformities. Tbx5 is evolutionarily conserved, and zebrafish deficient for Tbx5 mimic many aspects of the HOS phenotype. However, how Tbx5 downstream activities control cellular functions and tissue morphogenesis has remained elusive. Recently, our lab identified a novel Tbx5 paralog (Tbx5b) in zebrafish. My preliminary data demonstrate that Tbx5a (previously Tbx5) and Tbx5b have distinct expression profiles throughout most of heart development, suggesting that these paralogs have evolved specialized functions that constitute the single Tbx5 of other vertebrates. This natural model of functional variation provides the unique opportunity to gain a deeper under standing of the distinct developmental processes of cardiogenesis. Thus, I hypothesize that Tbx5b has specialized regulatory functions at the cellular level that control morphogenesis into cardiac chambers and outflow tract, including valves. Determining these cellular activities downstream of Tbx5b is critical for extending our knowledge of the full range of Tbx5 functions in mammals and thus developing novel treatments for CHD.

3.27 Tbx1 interacts with the BAF chromatin remodeling complex.

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The T-Box transcription factor Tbx1 is required for a number of developmental processes, including the regulation of proliferation, differentiation and, possibly migration of cardiac progenitors of the second heart field (SHF). However, the mechanisms by which Tbx1 exerts its transcriptional functions are not well understood. Here we tested interactions between Tbx1 and chromatin remodelers and histone modifiers and asked how these interactions affect the transcription of target genes. We have identified a novel target of Tbx1, *Wnt5a*, a gene encoding for a ligand of the non canonical Wnt pathway. *Wnt5a* is expressed in the SHF, and is required for cardiac outflow tract development. Using genetic crosses, we demonstrated that *Wnt5a* and *Tbx1* interact genetically. *Wnt5a* harbors two Tbx1-responding enhancers. We used these enhancers to explore the mechanisms of Tbx1 function. We established that Tbx1 interacts directly with Baf60a, a subunit of the BAF chromatin remodeling complex. Increased dosage of Tbx1 enhances the occupation of Baf60a onto the *Wnt5a* gene in vivo and in tissue culture. Knock-down of Baf60a abolishes the ability of Tbx1 to regulate the *Wnt5a* gene as well as other targets. Furthermore, we found a physical interaction with the histone methyltransferase

Setd7 and the enrichment of H3K4 monomethylation status on the target loci after increased dosage of *Tbx1*. Our data indicate the importance of Baf60a for Tbx1-induced regulation of *Wnt5a* and perhaps other target genes. Hence it is reasonable to speculate that this BAF subunit is a key cofactor for Tbx1 function in cardiac progenitors.

3.28 ChIP-seq identification of downstream targets of Nkx2.5 during heart development

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Nkx2.5 function has been shown to be essential at various stages of mouse heart development, in addition to playing an important role in physiology of the newborn heart. Moreover, human NKX2.5 mutations have been suggested as the underlying cause of a variety of congenital heart diseases. Despite extensive analyses, the downstream effectors mediating Nkx2.5 function during heart development remain largely unknown. In order to identify mediators of Nkx2.5 function in vivo we used chromatin immunoprecipitation followed by massive parallel sequencing (ChIP-seq) in the E11.5 mouse heart. We were able to map several thousands of *in vivo* binding sites for Nkx2.5 in the mouse genome at that stage. Interestingly, a large number of the regions bound by Nkx2.5 have already been shown to be bound by the enhancer associated protein p300. Thirty-seven of the regions binding both Nkx2.5 and p300 have been previously shown to have cardiac-specific enhancer activity in vivo in transgenic mouse reporter assays suggesting that these proteins act together to regulate a subset of cardiac genes. To discriminate between functional and non-functional binding, we compared this data set with changes in gene expression, using expression array analysis of a hypomorphic model of Nkx2.5 expression. With this combined approach, we identified 116 genes dysregulated in the Nkx2.5 hypomorph at E11.5 that contain a Nkx2.5 binding site, strongly suggesting that they are direct targets of Nkx2.5. Surprisingly, more than 30% of these direct targets have a function related to the regulation of energy metabolism, suggesting that Nkx2.5 acts as a key regulator of the metabolic processes during cardiac development.

3.29 A meta-analysis of global gene expression and interaction in heart development of mouse

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Heart development is a complicated biological process that involves the activations and interactions of thousands of genes that form a gene regulatory network. To unravel this gene network is highly challenging because we have limited knowledge about gene functions and gene-gene interaction and it is computationally difficult to infer from the high-dimensional genomic data. The fast advancement of high throughput biotechnology is generating a wealth of genome-wide information regarding gene expression, DNA binding, DNA and RNA sequences, and epigenetic effects that provides an unprecedented opportunity to understand gene functions and interactions during heart development. In this project, we have integrated gene expression profiles from multiple developmental stages and ChIP-Seq data for a few important transcriptional factors that are involved in heart development in mouse. A meta-analysis was conducted to illustrate gene differentially expression at different developmental stages and to infer the gene interactions and activated pathways for heart development.

Section 4: Neural Crest and Vascular Development

S4.1 Early neural crest-restricted Noggin over-expression results in congenital craniofacial and cardiovascular defects

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Noggin is a secreted BMP antagonist that binds BMP ligands and prevents their activation of receptors. Noggin (in concert with Wnts, FGFs, Delta/Notch) plays a key upstream role in neural crest cell (NCC) induction by generating a BMP morphogenic gradient that activates a cascade of transcription factors culminating in the

ultimate steps of NCC differentiation. NCC derivatives are absolutely essential for cardiac outflow tract (OFT) remodeling, specifically during OFT septation and patterning of the great vessels that exit the heart.
Using Cre/loxP, constitutive Noggin expression was induced within NCC via *Pax3^{Cre}* (very early within neural tube), *Wnt1-Cre* (early within neural tube) or *Peri-Cre* (post-migratory) drivers. Consistent with its *in vitro* role in negatively regulating BMP signaling, Westerns confirmed *Noggin* overexpression significantly suppressed phosphorylation levels of Smad1/5/8 but left Smad2/3 unaffected *in vivo*. Moreover, *Wnt1-Cre* and *Pax3^{Cre}*-mediated suppression of BMP signaling resulted in subsequent loss of NCC-derived craniofacial, pharyngeal and OFT cushion tissues. Increased cell death was observed in pharyngeal arch NCC and DRG (although cell proliferation was unaltered), but not within the OFT itself. Lineage mapping demonstrated that NCC emigration was not affected in either *Pax3^{Cre};Noggin* or *Wnt1-Cre;Noggin* mutants, but that subsequent colonization of the OFT was significantly reduced in *Wnt1-Cre;Noggin* and completely absent in *Pax3^{Cre};Noggin* mutants. Further, although *Wnt1-Cre;Noggin* mutants are viable until birth, *Pax3^{Cre};Noggin* mutants all die by E14. Conversely, *Peri-Cre* mediated suppression of BMP signaling in post-migratory NCC had no effect and mutants are viable at birth. Taken together, these data show that conditional BMP inhibition within the NCC may exhibit different effects dependent upon timing and that tightly regulated TGFβ superfamily signaling plays an essential role during craniofacial and cardiac NCC survival *in vivo*.

S4.2 The Role of Fibronectin - Integrin Signaling in Pharyngeal Microenvironment in Modulation of Cardiac Neural Crest Cell Development

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Patterning of Pharyngeal Arch Arteries (PAAs) is a critical process in embryonic cardiovascular development. Alterations that perturb this process cause cardiovascular abnormalities such as human DiGeorge Syndrome. Our lab previously discovered defective development of CNCCs in global fibronectin (FN)-null or integrin a5-null mutants. In order to determine the roles of FN synthesized by the pharyngeal microenvironment during CNCC development, we conditionally ablated FN or the α5 subunit of its integrin receptor using *Isl1^{Cre}* strain of mice. We found that conditional inactivation of FN or integrinα5 using *Isl1^{Cre}* cause embryonic/neonatal lethality. Both of these conditional mutants display various cardiovascular and glandular anomalies associated with defective PAA and CNCC development. Moreover, histological analyses reveal defective development of ventricular myocardium and muscular ventricular septum, phenotypes dependent on the proper development of the mesodermal cardiac progenitors. Taken together, these phenotypes suggest that FN-integrin α5 signaling to distinct tissues comprising pharyngeal microenvironment play critical roles in PAA development by modulating interactions between CNCCs and non-CNCCs. To understand the basis of these phenotypes, we have investigated the contribution of CNCC to PAA formation/remodeling and found that FN promotes survival of CNCCs and cardiac progenitors. In addition, FN synthesized by pharyngeal tissues regulates CNCC navigation. We are investigating possible roles of FN and integrin α5-regulated signaling in regulation of morphogens, guidance molecules and growth factor signaling to tissues comprising the pharyngeal microenvironment. Taken together, our studies provide important insights into how tissue microenvironment regulates morphogenesis of complex organs from diverse populations of progenitors.

S4.3 Slit3-Robo1/2 signalling controls cardiac innervation and ventricular septum development by regulating neural crest cell survival

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The Slit-Robo signalling pathway has been shown to have pleiotropic effects in Drosophila heart development, however, its involvement in mammalian heart formation is yet largely unknown. Here, we analysed the role of this signalling pathway during murine heart development. We observed extensive expression of both Robo1 and Robo2 receptors and their ligands, Slit2 and Slit3, in and around the developing heart. Analysis of mice lacking Robo1 revealed membranous ventricular septum defects and decreased cardiac innervation, while animals lacking Robo2 did not display such defects. However, the combined absence of both Robo1 and Robo2 caused increased incidence and severity of membranous ventricular septum defects. Mice lacking the Slit1 and Slit2 ligands did not reveal any abnormalities, but Slit3 mutants recapitulated the membranous ventricular septum

defect. Interestingly, the density of cardiac innervation was increased in Slit3 mutants. The combined absence of the membranous ventricular septum and innervation defects suggested a defect in cardiac neural crest contribution. Detailed cell counts showed a reduction in neural crest cell contribution to the outflow tract. Further analyses indicated increased apoptosis in the neural crest, just before entering the heart, suggesting that cell death underlies its reduced contribution to the heart. Our data indicate a novel role for Slit3-Robo1/2 interaction in cardiac neural crest survival and, thereby, in the formation of the membranous ventricular septum and cardiac innervation.

S4.4 The neural crest contributes to coronary artery smooth muscle formation through endothelin signaling

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Endothelin-1 (ET1) /Endothelin A receptor (ETAR) axis has important roles in regulating cardiovascular homeostasis and organ development. We have previously reported that ET1 and ETAR knockout mice display craniofacial defects and aortic arch malformations. We also found their expressions in the developing coronary artery smooth muscle cells (CASMCs), however their roles in coronary artery development had been unknown. Here we show that ET1/ETAR axis insufficiency disturbs coronary artery development. Fetal coronary angiography revealed some septal branches were abnormally enlarged in both ET-1 and ETAR KO mice at embryonic day 17.5 (E17.5) and the branching pattern of the septal branch was also altered in both KO mice. Immunohistochemical analysis revealed partial lack of CASMCs in KO mice and serial analysis showed these malformations were occurred at the remodeling stage of coronary arteries. Each phenotype appeared in the septal branch-specific manner, then we hypothesized these segment specificities stood on the variety of cell sources of CASMCs. Fate mapping using Wnt1-Cre mice suggested that lacked CASMCs were specifically derived from the neural crest cells. To analyze the contribution of neural crest cells to CASMCs in detail, we used quail-chick chimera technique and neural crest ablation models. Quail-chick chimera with a particular region of the neural crest recaptured the preferential distribution pattern to the interventricular septum region. Chick ablation models also showed coronary artery malformations similar to those in ET-1 and ETAR KO mice. These results indicate novel contribution of the neural crest to coronary artery formation partly through endothelin signaling.

S4.5 CASTOR directly regulates a novel Egfl7/RhoA pathway to promote blood vessel development and morphogenesis

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The formation of the vascular system is essential for embryonic development and homeostasis. Consistent with recent genome wide association studies implicating a genetic link between the transcription factor CASTOR (CST) and high blood pressure and hypertension, we demonstrate here that CST has an evolutionarily conserved function in blood vessel formation; in the absence of CST, Xenopus embryos fail to develop a fully branched and lumenized vascular system and human endothelial cells (HUVECs) lacking CST display dramatic defects in adhesion/contractility and cell division. Using chromatin immunoprecipitation (ChIP), we go on to identify Epidermal growth factor-like domain 7 (Egfl7) as a direct transcriptional target of CST. We demonstrate that CST is required for expression of Egfl7, that CST is endogenously bound to the Egfl7 locus in the developing embryo, and that depletion of EGFL7 phenocopies CST depletion in whole embryos and in HUVECs. We further show that the adhesio n/contractility abnormalities due to depletion of CST in HUVECs can be rescued by the reintroduction of EGFL7. Critically, we have demonstrated that CST and EGFL7 modulate endothelial cell shape and contractility through the small GTPase RhoA. Collectively, these data support a mechanism whereby CST directly regulates its transcriptional target Egfl7 to promote RhoA-mediated endothelial cell shape and adhesion changes to establish the vasculature and hence, these studies provide insight into the cellular and molecular basis for vascular disease associated with the loss of CST.

S4.6 Nephronectin regulates axial vein morphogenesis in zebrafish

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Angiogenesis is the development of new vessels from pre-existing vessels. This is a critical morphological event both in organ development as well as in diseases. Like in other vertebrates, in zebrafish vessels form a complex network in order to fulfill tissue oxygen demands. Development of complex vascular networks is dependent on the directional migration of groups of endothelial cells, which is called angiogenic sprouting. Here we have demonstrated that in zebrafish the extracellular matrix protein, nephronectin, is transiently expressed in the caudal vein plexus forming region at the time of caudal vein sprouting at around 30 hours post fertilization (hpf). Morpholino-mediated nephronectin depletion resulted in the malformation of the caudal vein plexus and the ventral vein and in the frequent loss of inter-segmental veins. Time-lapse analysis from 28 hpf to 40 hpf indicated a decreased in the frequency of caudal vein sprout formation in nephronectin morphants. In addition, existing sprouting appeared multi-directional indicating a navigation problem. Biochemical analysis demonstrated that nephronectin is able to bind to the integrin αV/β3 heterodimer. Importantly, integrin αV and nephronectin expression overlapped in the region of the caudal vein plexus. Moreover, morpholino-mediated integrin αV knockdown in zebrafish phenocopied nephronectin depletion. Taken together, our data indicate that nephronectin regulates directional sprouting of the axial vein in zebrafish, which might be via integrin αV.

4.7 Coordinated patterning mechanisms of blood vessels and peripheral nerves

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Anatomical proximity and close patterning of nerves and blood vessels suggest that there is interdependence between the two networks. The first such indication of this interplay is the responsiveness of vascular development to signals secreted by peripheral sensory nerves in the developing skin. We suggests a coordinated mechanism in which nerve derived-signals is responsible for vascular patterning signal to recruit vessels to align with nerves, and for arterial differentiation in the nerve-associated vessels. Interestingly, we discovered a reciprocal guidance event in the patterning of peripheral sympathetic nerves in the developing heart. Our whole-mount imaging approaches revealed that the pattern of large-diameter coronary veins influences the pattern of sympathetic innervation in the developing heart. Further genetic studies and *in vitro* organ culture experiments demonstrated that coronary veins serve as an intermediate template that guides distal sympathetic axon projection via local signal by coronary vascular smooth muscle cells. Our results suggest that target organs possess unique and stereotypical patterns of innervation, mediated by tissue sub-structures, such as coronary veins in the heart, that are adapted to complex organ structure and physiology.

4.8 Targeted non-invasive occlusion of pharyngeal arch arteries during avian cardiac morphogenesis

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The role of hemodynamics in outflow tract and pharyngeal arch artery morphogenesis is poorly understood. Through the use of two- photon microscopy guided femtosecond laser pulses, we nucleated and controlled the growth of microbubbles within outflow vessels without disturbing surrounding tissues. These bubbles temporarily occluded the vessel, during which time a stable occlusion could be formed by ablating the circulating thrombocytes that accumulated behind the bubble. These clot-like structures then persisted. Using this approach, we examine the effects of PAA vessel occlusions on embryonic viability, hemodynamic rearrangement, and downstream outflow tract morphogenesis. We determine that occlusion of the right IV pharyngeal arch artery caused lethality in 99.5% of embryos by day 6 (HH28/29), while occlusion of the left IV arch artery was significantly less lethal. In each case, blood flow was redistributed to the III and VI PAA and vessel diameters were altered significantly. With right IV PAA occlusion, the right III PAA artery increased 47% in diameter while the right VI decreased 100%. Interestingly, ultrasound derived blood velocity did not change in the right III PAA but significantly increased in the right VI. No changes in diameter or velocity were seen in the contralateral PAA. When both IV PAA were occluded, both PAA vessels changed in diameter, resulting in an overall decrease in ouflow lumen area. These results support that PAA hemodynamics are important contributor to embryonic development.

4.9 ORIGIN AND MIGRATION OF ARTERIAL AND VENOUS PROGENITORS FOR THE MAJOR AXIAL VESSELS

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During the formation of the major axial vessels, the dorsal aorta (DA) and the posterior cardinal vein (PCV), vascular endothelial progenitor cells (angioblasts) adopt an arterial or venous identity prior to circulation. However, how angioblasts establish their arterial or venous fates and how they migrate to assemble into the major axial vessels remains controversial. Using the zebrafish as the model organism, we performed single cell fate mapping and time lapse imaging to investigate the origin of angioblasts and their migratory behavior during the assembly of the axial vessels. Our results show that in contrast to the current model, the progeny of single labeled angioblasts can contribute to both the DA and the PCV, and that the arterial or venous identity of the endothelial progenitors is not determined until they migrate towards the midline. We show that angioblasts originate at different times in two distinct locations, a medial line and a previously unobserved lateral line, both of which exhibit different biases in arterial-venous contribution. Based on cell tracking results, most angioblasts migrate directly to their arterial and venous positions rather than assembling into a single common precursor vessel as previously suggested. Using photoactivatable morpholinos, we show that the function of the *Etv2*/*Etsrp* transcription factor, a known regulator of early vasculogenesis, is required earlier for arterial differentiation than for venous, which corresponds to the timing of the medial and lateral angioblasts migration. Our results lead to a revised model for arterial-venous differentiation, and will help to dissect the mechanim for arterial-venous specification.

4.10 Vessel diameter increase during remodeling of the mouse yolk sac occurs via vascular fusion and endothelial cell recruitment

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For over the past one hundred years, it has been posited that blood flow regulates the remodeling of vessels, and only recent studies have determined that hemodynamic forces imparted by blood flow are required for vascular remodeling. However very little is known about the cellular events that promote vessel diameter increase during remodeling, and how hemodynamic force triggers these events. To assess the cellular events of remodeling, we performed live imaging of cultured mouse embryos labeled by two endothelial cell transgenic reporter lines (*Flk1-H2B::eYFP* [nucleus] and *Flk1-myr::mCherry* [membrane]). Our data have revealed that vessels of the mouse yolk sac increase their diameter during remodeling via localized recruitment of endothelial cells to growing vessels, and via vascular fusion, which also acts to refine vessel branches. Hemodynamic force is a critical factor driving vessel diameter increase as only vessels exposed to high amounts of blood flow undergo vessel fusion. Also, arteries exposed to high blood flow exhibit directional migration of endothelial cells upstream of the direction of blood flow to hierarchical vessels, and exhibit directional migration of endothelial cells towards high flow arteries from low-flow/interconnected vessels. Whereas, vascular fusion events are absent in embryos exposed to reduced blood flow (Myosin light chain 2 α mutants), and endothelial cells in these embryos do not exhibit a directional migratory behavior. Taken together, these results directly show in a mammalian system the cellular changes that occur during vessel diameter expansion in response to hemodynamic force.

4.11 med23, a subunit of transcriptional mediator complex, regulates embryonic angiogenesis and is essential for embryonic cardiovascular development

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med23, a subunit of transcriptional mediator complex, is demonstrated co-regulating expression level of various groups of genes in ES and MEF cells with members of SPF family. While other candidates binding to med23 remain to be identified. med23 whole body mutant murine embryos was generated to study the role of med23 in embryonic development. Knock-out embryos are lethal at early stage with defects at neural and cardiovascular system. To further investigate the role of med23 in cardiovascular development, we generated med23 conditional knock mice and deleted med23 in endothelial cells specifically with crossing female med23 f/f to male med23 f/+::Tek-Cre mice. Majority of cKO med23 mutant embryos died at two stages. The early group is lethal between E9.5 and E11. Possible lethal reason is failure formation of mature vascular system at placenta

and yolk sac. The later group is dead between E12.5 and E14 with sever hemorrhage at whole body, especially at head region, decreased angiogenesis at embryo body and yolk sac and decreased integrity of blood vessels. Normal arterial-venous differentiation was observed in mutant embryos and normal cardiac development was showed in majority of mutant embryos. After contrasting angiogenesis relative genes expression in head region of control and mutant embryos, we detected down-regulation of Notch signaling ligand Jag1 and downstream genes. The phenotype of Jag1mutant embryos specific in endothelial is similar with med23, displaying decreased angiogenesis, dilated small blood vessels, shrink large blood vessels and hemorrhage. We presume med23 regulates angiogenesis process via regulating Notch pathway. It is reported Jag1 regulates smooth muscle differentiation as well. We also identified down-regulation of αSMA gene expression at yolk sac. To further investigate details of phenotype and mechanism, In vitro experiments of endothelial cell assay and in vivo cell trace experiments will be operated.

4.12 Regulatory mechanisms of Semaphorin 3C signaling essential for interaction between cardiac neural crest and the second heart field

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The cardiac neural crest (CNC) and the second heart field (SHF) play key roles in development of the cardiac outflow tract (OFT), and their interaction to form the outflow tract septum is essential for establishment of pulmonary and systemic circulation in higher vertebrates. Recently, we showed that GATA6 directly regulated the semaphorin 3C (SEMA3C) signaling that is essential for interaction between CNC and SHF during the development of the OFT, and that mutations of GATA6 disturbed the activation of SEMA3C, resulting in persistent truncus arteriosus. We further delineated promoter/enhancer of Sema3c and identified conserved regulatory elements for Fox transcription factors within 1kb upstream region and for T-box factors within 2kb downstream in the 3'UTR, respectively. Interestingly, Foxc1 and Foxc2 activated Sema3c in the OFT region through direct bindings to their regulatory elements. On the other hand, Tbx1 negatively regulated the transactivation of Sema3c by G ata6. Moreover, the expression of Sema3c was ectopically expanded in pharyngeal mesenchyme in mice with Tbx1 hypomorphic alleles, while it is normally restricted to the SHF in the pharyngeal mesoderm. These data suggest that Tbx1 may restrict the Sema3c expression in the SHF for proliferation of progenitor cells to form the OFT, and Gata6 and Foxc1/c2 may activate the Sema3c expression in these cells in a process of migration and differentiation into the OFT. Further temporal-spatial molecular events regulating the Sema3c signaling underlying interaction between CNC and SHF will be discussed.

4.13 SHP2, NEURAL CREST, AND NEONATAL DEATH

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Src-homology protein tyrosine phosphatase 2 (Shp2) plays diverse roles in many human diseases including LEOPARD and Noonan syndromes, both of which are characterized by neural crest (NC) abnormalities such as craniofacial malformations and congenital heart defects. Shp2 is thought to mediate its functions through a plethora of signaling cascades including Extracellular Regulated Kinases (ERK) 1 and 2. We hypothesize that abrogation of downstream ERK1/2 signaling in NC lineages is primarily responsible for the phenotypes observed. In order to investigate the role of Shp2 and downstream mediators within NC cells, we generated a unique conditional knockout (cKO) of *Shp2* in post-migratory NC using a novel *Periostin-Cre* line engineered in our lab. Unexpectedly, *Shp2* cKOs are indistinguishable from control littermates at birth and exhibit no gross structural cardiac anomalies. However, around 1-2 weeks after birth, *Shp2* cKOs drop off the growth curve and develop electrocardiogram abnormalities. Significantly, 100% *Shp2* cKOs die within 3 weeks after birth. Lineage mapping using a *LacZ* reporter mouse (*ROSA26r*) of Cre expression revealed significant hypoplasia of the sympathetic and sensory nervous systems from 3+ days after birth. Specifically, the neural plexus innervating the heart and the sympathetic chain were reduced. Molecularly, *Shp2* cKOs exhibit lineage-specific suppression of activated phospo-ERK1/2 signaling, but not of other downstream targets of Shp2 such as AKT. These preliminary data suggest that the hypoplasia of the sympathetic and peripheral nervous system, the ECG abnormalities, and our neonatal death phenotype may all be directly mediated via a loss of pERK in post-migratory NC lineage.

4.14 Role of endocytosis by the neural crest cells in cardiovascular development

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Cardiac neural crest cells (CNCCs) are required for proper remodeling of the pharyngeal arch arteries (PAAs), correct alignment, and septation of the cardiac arterial pole. CNCCs are thought to regulate signaling in the pharynx impacting PAA and secondary heart field (SHF) development. However, it is unknown how this is accomplished. Signaling pathways involved in cardiovascular development, including Notch, SHH, and FGF, rely on endocytosis of their respective ligands. Here we test the hypothesis that endocytosis by the CNCCs is required for normal cardiovascular development. An inhibitor of clathrin-dependent endocytosis, Pitstop2, was applied to chick embryos. Embryos displayed double outlet right ventricle (DORV), indicating disrupted development of the SHF, as well as abnormal persistence of the fifth and left fourth PAAs, reminiscent of human double aortic arch. Surprisingly, PAA defects occurred most frequently in embryos treated prior to CNCC migration, long before ve ssel remodeling occurs. Premigratory CNCCs were targeted by electroporation with either dominant negative Dynamin (dnDyn) or dnRab5a to block endocytosis or vesicle trafficking. Experimental embryos showed DORV and PAA remodeling defects similar to Pitstop2 treated embryos. Smooth muscle differentiation within the PAA walls was abnormal. To determine which signaling pathways are impacted by endocytosis knockdown, RNA was extracted from individual pharynxes for RNA-Seq. This is the first animal model of double aortic arch, and provides a framework for investigating the mechanism by which the CNCCs remodel the paired PAAs into the asymmetric great arteries. Altogether the evidence indicates a critical role for endocytosis in proper orchestration of cardiovascular development.

4.15 A New Perspective on the Origin and Derivatives of Cardiac Neural Crest in Zebrafish

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Cardiac neural crest cells (NCCs) in mouse and chick give rise to the smooth muscle of the aortic arch arteries, form the aorticopulmonary septum and the cardiac ganglia. Zebrafish have unseptated hearts and may not have cardiac NCCs. We hypothesize that cardiac NCCs contribute to the gill arch arteries and ventral aorta but not to other heart structures. Two previous studies in zebrafish reported the origin of cardiac NCCs and that they gave rise to myocardium, a fate not observed in chick and mouse. Neither study took a direct approach of tracing known cardiac NCCs. Using the neural crest transgenic *Tg(sox10:egfp)*, we used uncageable rhodamine to determine the fate of gfp-positive NCCs as they migrated from the neural tube. We refined the map of the NCCs that contribute to the branchial arches but found no contribution to the myocardium at 72hpf, as has been previously reported. We showed that cranial NCCs and cardiac mesoderm were in close proximity (3-5 cell diameters apart) as NCCs migration begins. Therefore, the previous studies may have spuriously labeled cardiac mesoderm. Using a *Tg(Sox10:Cre)* reporter line, which permanently labels cells that express sox10, we found sox10 positive cells in the heart at 96hpf after all myocardial cells were added. At 120hpf, we found sox10 positive cells in the ventral aorta, similar to NCCs seen in the aortic arch arteries of mice and chicks. Cells at 96hpf are likely contributing to innervation of the heart, as seen in mouse and chick models.

4.16 Combinatorial Actions of Retinoic Acid and Sonic Hedgehog in Cardiac Neural Crest

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The vitamin A derivative retinoic acid (RA) acts with other embryonic signals to control growth and patterning. As the RA and Sonic hedgehog (Shh) pathways synergistically control neural progenitor cell survival and differentiation, we hypothesized similar effects may occur on ectomesenchymal neural crest cells (NCCs). RA deficiency in Raldh2-/- embryos produces aplasia of the 3rd-6th pharyngeal arches. While NCCs form and initiate their migration, few cells migrate past the defective pharyngeal region towards the heart outflow tract. Hox genes are regulated by both RA and Shh signaling. Early NCC migration and guidance relies on Hoxa1 and Hoxb1, whereas genes from paralogy groups 2 to 4 are believed to convey pharyngeal patterning information. Hoxa1, Hoxa3/d3, Hoxa4/d4, and Hoxb5 levels in the posterior pharyngeal arch region were all reduced under RA deficiency and these genes were resistant to activation under high level-retinoid treatment. Daily injection of the Shh inhi bitor cyclopamine at E7.5-E8.5 effectively blocked the hedgehog targets Ptch1 and Gli1. Combined Shh inhibition and Raldh2 deficiency reduced the quantity of NCCs migrating into the 3th-6th aortic arch region.

These NCC depletions were also observed in Nkx2.5-Cre-deleted Shh/Raldh2 double mutants and in explant cultures in which both pathways were blocked. We propose a two-step model in which (i) RA induces multiple Hox paralogy group expression, and is required for a Hox "code" providing positional identity for caudal pharyngeal arch patterning; (ii) combined RA and Shh actions are required for ectomesenchymal NCC progenitors survival, prior to their arrival in the pharyngeal region.

4.17 Roles of neural crest-derived fibronectin in cardiovascular development

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Fibronectin (FN) is essential for vertebrate development, however the function of FN in orchestrating developmental processes is not well understood. Our previous study has shown that cardiac neural crest (CNC) cell numbers are significantly deficient in FN global null embryos due to defective proliferation and survival of CNC progenitors, while migration of the CNC is not significantly altered. FN and multipotent neural crest are unique vertebrate features, and work from our lab showed that FN gene expression is upregulated in the neural crest before and immediately after neural crest cells exit from the dorsal neural tube. In order to examine the role of FN in neural crest development, we used two different lines of mice to ablate FN synthesized by the neural crest, P3Pro-Cre (neural crest) and AP2a$^{IRES\text{-}Cre}$ (neural crest and surface ectoderm). The phenotypes of the conditionally mutant embryos were similar when either of the above strains was used, indicating that the observed defects were due to the ablation of FN in the neural crest. We found that conditional ablation of FN using either of the above strains leads to embryonic lethality and severe cardiovascular abnormalities, including intra-cardiac cartilage, retroesophageal right subclavian artery, ventricular septum defect, persistent truncus arteriosus, double outlet right ventricle, and overriding aorta. Although initial migration of neural crest cells occurred normally in the mutant embryos, some FN mutants exhibited decreased numbers of CNC cells compared with their littermate controls. At E11.5, mutant embryos were deficient in neural crest derived smooth muscle cells around the right fourth aortic arch artery. Further studies will continue to investigate molecular roles of the neural crest cell-synthesized FN in neural crest development and cardiovascular morphogenesis.

4.18 Correlation of Great Artery Growth with Hemodynamic Wall Shear Rate in the Developing Mouse Fetus: a Study using Ultrasound Imaging, EFIC Reconstructions and Computational Modeling.

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Introduction: Adverse fluid mechanical environment of the fetal cardiovascular system has been hypothesized to play a role in structural heart malformations. We present a study of the geometry and fluid mechanics of fetal great arteries during normal fetal development from embryonic day 14.5 (E14.5) to near term. **Methods:** Ultrasound bio-microscopy (UBM) was used to measure blood velocity of the great arteries. Subsequently, specimens were cryo-embedded and sectioned using episcopic fluorescent image capture (EFIC) to obtain high-resolution 2D serial image stacks, which were used for 3D reconstructions and quantitative measurement of great artery and aortic arch dimensions. EFIC and UBM data were input into computational fluid dynamics (CFD) for modeling hemodynamics. **Results:** In normal mouse fetuses between E14.5-18.5, ultrasound imaging showed gradual increase in blood velocity in the aorta, pulmonary trunk, and descending aorta. Measurement by EFIC imaging showed a similar gradual increase in cross sectional area of these vessels. CFD modeling showed great artery hemodynamic wall shear rate to be 2900-3300 sec^{-1} at peak flow. Average wall shear rate remained at this level despite vessel growth over these stages. **Conclusion:** We observed the maintenance of a constant wall shear rate during mid to late mouse fetal development. These results suggest a homeostatic sensing mechanism that may link hemodynamic shear to the regulation of vessel growth. Further, insights into fetal cardiovascular fluid mechanics may help elucidate whether fetal cardiovascular surgery may have benefit for patients with congenital heart disease. (Study supported by U01-HL098180)

4.19 The role of Forkhead Box transcription factors Foxc1 and Foxc2 in myocardial wound repair

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Summary: Forkhead Box (Fox) transcription factors are essential for regulating cell growth, proliferation, differentiation, and longevity. Among the large subclasses (FoxA to FoxS) of Fox transcription factors, Foxc1

and Foxc2 are known as important regulators for cardiovascular development. However, the role of Foxc1 and Foxc2 in pathological neovascularization involving ischemic condition remains still unknown. Here, we employed a mouse myocardial infarction (MI) model to study the role of Foxc1 and Foxc2 in cardiac tissue repair following ischemic insult. **Methods**: Foxc1 and Foxc2 heterozygous mutant (+/-) and wild-type mice were used for this study, and MI was induced surgically by ligating the left anterior descending coronary artery. To examine heart functions, we measured fractional shortening and ejection fraction at day 7, 14 and 28 after MI using echocardiography. After 4 weeks post-surgery, histological sections of heart samples were obtained to observe fibrosis with Masson trichrome staining. **Results**: There was no significant difference in infarction areas between Foxc1+/- and wild-type mice, and the ejection fraction and fractional shortening did not differ between the two groups at each time point. In contrast, echocardiographic measurements of left ventricular ejection fractions and fractional shortening were reduced in Foxc2+/- mice after MI compared to wild-type mice, whereas cardiac fibrosis was increased in Foxc2+/- mice. Most significantly, Foxc2+/- mice had a drastic reduction in capillary vessel formation in the ischemic border zone after MI compared to wild-type mice. **Conclusions**: Our results indicate that Foxc2 is more involved in neovascularization during ischemic tissue repair after MI than Foxc1.

4.20 Intrinsic non-mesothelial origin of endothelial cells in the avian intestine.

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The proepicardium of the heart contributes at least a subpopulation of coronary endothelial cells to the developing heart. The avian intestine does not derive its mesothelial lining from a proepicardial-like structure. Instead, mesothelial progenitors are resident and broadly distributed throughout the intestinal primordium. We investigated the mechanisms of intestinal vascular formation to determine if similar variation could be observed between the heart and intestine in endothelial origins. We labeled intestinal mesothelial progenitors with a replication incompetent retrovirus and allowed the embryos to develop for up to two weeks. While we observed vascular smooth muscle cells and pericytes among the progeny of labeled mesothelial progenitors, we did not identify any endothelial descendants. We also transplanted quail splanchnopleure prior to mesothelial formation into the coelomic cavity of chick embryos. The grafted splanchnopleure generated a gut tube complete with a mesentery and vasculature. We determined that endothelial cells of the graft were derived primarily from the graft and not from the host. Injections of lectin and a vital dye directly into the heart of HH14-17 quail embryos also demonstrated the endothelial plexus of the splanchnopleure is in communication with the systemic vasculature. Furthermore, intracardiac injection of a replication incompetent retrovirus at HH14-17 resulted in labeled endothelial cells throughout the vascular tree of the intestine demonstrating endothelial cells of the early splanchnopleure are remodeled to generate the mature vasculature. Thus, we conclude the majority of avian intestinal endothelial cells are derived from remodeling of the primitive endothelial plexus of the splanchnopleure.

4.21 A comprehensive timeline of endothelial development in the small intestine of the quail embryo

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The early embryonic gut is a tube composed of splanchnic mesoderm and endoderm. Many studies have investigated specific tissue layers or time points in gut development. However, an inclusive timeline of endothelial cell development and the simultaneous events that occur throughout small intestine development is lacking. We utilized immunofluorescence, morphometric analysis, histology, and transgenic quail embryos to examine the relationship of endothelial plexus formation to mesothelial formation, mesenchymal growth, and smooth muscle morphogenesis. Using Tg(*tie1*:H2B-eYFP) quail embryos, the nuclei of endothelial cells are first visualized in the mesenchymal space at E2.1, between two basement membranes, lining the endoderm and splanchnic mesoderm, in the open gut tube. At E6, two concentric rings of endothelial cells are observed, at the same time point the gut tube closes, and when both a mesothelium and visceral smooth muscle are first observed. At E11, the major blood vessels, circumferentially covering the midgut, are first detected, and smooth muscle cells develop around the YFP+ cells, indicating muscularization of the vessels. We observed endothelial cells in the villi at E14, at which smooth muscle actin staining is also visualized for the first time in the villi. Taken together, these data provide a comprehensive timeline of endothelial development in the small intestine. Normally, developmental processes not correlated to one another may have both a temporal and morphological

relationship. This timeline will benefit both researchers examining intestinal development and clinicians studying congenital syndromes that may originate from a combination of disrupted developmental processes.

4.22 SEMAPHORIN3D PATTERNS THE PULMONARY VEINS

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A fundamental aspect of mammalian and avian cardiopulmonary physiology is the complete separation of systemic and pulmonary circulations. Establishment of this highly efficient oxygen delivery system requires both intracardiac septation and proper vascular patterning during embryogenesis. Total anomalous pulmonary venous connection (TAPVC) is a potentially lethal congenital heart disease that occurs when the pulmonary veins fail to connect normally to the left atrium, allowing mixing of pulmonary and systemic blood. In contrast to the broad knowledge of arterial vascular patterning, little is known about the patterning of veins. Here we show that the secreted guidance molecule Semaphorin 3d (Sema3d) is crucial for normal patterning of the pulmonary veins. Prevailing models suggest that TAPVC occurs when the midpharyngeal endothelial strand, the precursor of the common pulmonary vein, fails to form at the proper location on the ventral surface of the embryonic common atrium. However, analysis of Sema3D mutant embryos shows that TAPVC occurs despite normal formation of the midpharyngeal endothelial strand. Rather, the emerging venous plexus destined to form the pulmonary veins fails to anastomose uniquely with the properly formed midpharyngeal endothelial strand and forming endothelial tubes penetrate a boundary normally produced by Sema3d expression, resulting in aberrant connections. These results identify Sema3d as a critical pulmonary venous patterning cue and provide experimental evidence for an alternate developmental model to explain abnormal pulmonary venous connections.

4.23 A mouse fetus with a double lumen aortic arch malformation

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Double lumen aortic arches are rarely diagnosed in humans. They are considered to result from the abnormal persistence of a left 5th pharyngeal arch artery (PAA). Although the mouse is an important model for researching the genesis of PAA malformations, there is no documented case of a persisting 5th PAA in mice. This study aims at presenting a mouse fetus of the him:OF1 strain, which developed a double lumen aortic arch. In order to comprehensively characterize the malformation, we created surface rendered three-dimensional (3D) computer models of the great intrathoracic arteries with the aid of the "High-resolution episcopic microscopy" (HREM) technique. The voxel size of the 3D models was 1.07 x 1.07 x 2µm^3. The gross anatomy and the dimensions of the great intrathoracic arteries of the malformed fetus appeared to be normal with one exception. The segment of the aortic arch between the origin of the left common carotid artery and the left subclavian artery consisted of two channels. The length of the segment and the sum of the cross sectional areas of the two channels were approximately the same as the length and the cross sectional area of the corresponding one channeled aortic arch segment of normal fetuses. Our results show that mice, like humans, do show double lumen aortic arch malformations. They also show that this malformation does not affect the formation and remodeling of the arteries located proximally and distally.

4.24 Mouse embryos feature a 5th pair of pharyngeal arch arteries

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Recent studies do suggest that mouse embryos do not develop a 5th pair of pharyngeal arch arteries (PAAs). If true, the suitability of the mouse as a model for researching malformations of the great intrathoracic arteries would be degraded. The aim of our study was to evaluate whether mouse embryos do or do not develop a 5th pair of PAAs. We analysed the vascular phenotype of 30 mouse embryos aged 12-12.5 days post conception (dpc) with the aid of volume data and three-dimensional (3D) computer models generated with the "High resolution episcopic microscopy" technique. The embryos were of the Him:OF1 strain. The digital volume data

had a voxel size of 1.07x1.07x2 μm^3. In half of the embryos (15 of the 30) we detected a thick vascular channel, which connected the lumen of the 4th and the lumen of the 6th pharyngeal arch arteries. Nine embryos (30%) showed this channel unilaterally. Six embryos (20%) showed it bilaterally. According to descriptions of the human 5th pharyngeal arch artery and to descriptions of the 5th pharyngeal arch artery in rat embryos, we identified this vascular channel as the 5th pharyngeal arch artery. Our study demonstrates that mice do develop a of 5th pair of PAAs around 12.5 dpc. Hence it re-establishes the reputation of the mouse as an excellent model organism for researching the genesis of malformations of the great intrathoracic arteries of humans.

4.25 The phenotype of the pharyngeal arch arteries defines a new type of cephalothoracopagus embryos

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The presentation aims at providing a gross description of the phenotype of a cephalothoracopagus chick embryo of developmental stage 31 according to Hamburger Hamilton (HH) and a detailed metric analysis of its great intrathoracic arteries. It further aims at providing reference data, which define the normal dimensions of the great arteries at HH31. We used the high-resolution episcopic microscopy (HREM) technique for creating virtual three-dimensional (3D) models of 5 normally developed and one spontaneously developed cephalothoracopagus chick embryo. The models were employed for topologic analysis of the organs and blood vessels and for measuring the diameters of the lumina of the great intrathoracic arteries. The measurements were performed following a recently proposed measuring protocol. The cephalothoracopagus embryo showed two notochords, two spinal chords and two dorsal aortae. It had a single, four-chambered heart, a single respiratory tract, and a single upper alimentary tract. The topology of the pharyngeal arch arteries was normal. The dimensions of the lumina of the great intrathoracic arteries were similar to that of the control embryos. The phenotype of the cephalothoracopagus embryo does not match classical descriptions and cannot be entirely explained with any of the accepted theories. The embryo thus must be considered as having a yet unknown type of cephalothoracopagus malformation.

4.26 Molecular and Functional Profiles of VSMCs Based on Embryonic Origin

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Vascular smooth muscle cells (VSMCs) in the aorta are derived from two embryonic origins: the cardiac neural crest (CNC) and the mesoderm. The CNC contributes VSMCs to the ascending aorta and aortic arch (aAo) while the mesoderm contributes VSMCs to the descending aorta (dAo) distal to the ductus arteriosus. These two populations do not mix and remain compartmentalized through adulthood. Based on our preliminary and published data demonstrating distinct differences in VSMC gene expression and contractility, our hypothesis is that NC-derived VSMCs have molecular and functional characteristics distinct from mesoderm-derived VSMCs. Using myographic approaches, we established fundamental physiological differences between these two vessels in the embryo; the dAo is more contractile than the aAo. We conducted a microarray comparing these two regions of the embryonic aorta, and the results support our central hypothesis that aAo and dAo have distinct gene expression profiles. We have verified the expression of many of these genes with qRT-PCR. To confirm the functional difference between NC- and mesoderm-derived VSMCs, we have generated clonal VSMC lines from each region of the aorta and are assaying their contractile and migratory characteristics *in vitro.* Together these experiments will lead to discovery of molecular criteria to classify VSMCs and establish functional differences between VSMCs of distinct embryonic origins.

4.27 Tenascin-C may protect vascular wall as a molecular shock-absorber.

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Tenascin-C (TnC)is an extracellular glycoprotein, generally expressed at high levels during embryonic development in a spatiotemporal-restricted manner, sparsely detected in normal adult, but re-expressed in pathological condition in various tissue playing significant roles in tissue remodeling. During vascular

development, TnC is highly upregulated associated with smooth muscle/mural cell recruitment, however, the expression pattern is complex, often showing high sensitivity to mechanical stress. In normal adult mice, TnC is constitutively expressed in the abdominal aorta, but not in the proximal aorta. Biomechanical analyses revealed the significant reduction of flexibility of abdominal aorta of TnC knock out (TnC KO) mice to compare with that of the wild type, although TnC knock mice do not show distinct phenotype in the normal state. We created a mouse model of aortic stiffening by a combination of periaortic application of calcium chloride in the infrarenal aorta and systemic administration of angiotensin II (Ca+AngII). Ca+AngII treatment upregulated TnC expression in the upper aorta, and eventually induced marginal dilataion of the suprarenal aorta. In TnC KO, Ca+AngII treatment caused striking enlargement of the suprarenal aorta. Histological section of the enlarged suprarenal aorta showed a large medial dissection with "double barrel" appearance, while the wildtype showed only modest medial thickening and adventitial fibrosis. Transcriptome analysis indicated the reduced matrix deposition and exaggerated proinflammatory response in suprarenal TnC KO aorta before dissection. These results suggest TnC may work as a stress-activated molecular damper to keep the destructive stress response of vascular wall.

4.28 Valentine-like is a novel component of the CCM pathway and is essential for cardiovascular development in zebrafish.

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The cerebral cavernous malformation (CCM) pathway is required for normal cardiovascular development in zebrafish and mouse. We have previously shown that zebrafish embryos mutant for the CCM pathway genes *heart of glass* (*heg*), *santa* (*san*), or *valentine* (*vtn*) exhibit a massive dilation of the heart chambers and inflow tract and lack blood circulation. Recently, we have identified a novel, conserved gene bearing considerable sequence similarity to *vtn*, which we have named *valentine-like* (*vtnl*). Morpholino knockdown of *vtnl* confers a phenotype resembling that of the CCM pathway mutants; affected embryos have moderately enlarged atria and inflow tracts and lack circulation. Co-injection of *vtnl* and *san* morpholinos at concentrations that individually cause no phenotype results in characteristic cardiovascular defects, defining *vtnl* as an enhancer of the CCM phenotype. Overexpression of *vtn* can partially rescue *vtnl* morphant defects, suggesting that the two genes serve similar functions *in vivo*. *vtn* and *vtnl* both encode proteins containing phospho-tyrosine binding (PTB) domains, and we have shown in cell culture that Vtnl binds San in an interaction requiring the N-terminal region of San, which contains two NPxY motifs that are likely PTB targets. Mutations in the human homologs of *san* (CCM1), *vtn* (CCM2), and one other gene (CCM3) have previously been shown to cause cerebral cavernous malformations, a relatively common vascular anomaly. However, many CCM patients have no mutations in these genes. We propose that the human homolog of *vtnl*, C20ORF160, is an excellent candidate to be investigated for causative mutations in patients with cerebral cavernous malformations.

4.29 Hemogenic activity of the endocardium is regulated by Nkx2-5

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We have previously demonstrated that single cardiac progenitors expressing Flk1 Isl1, and Nkx2.5 give rise to both cardiac and endocardial/endothelial cell lineages during early murine cardiogenesis. Interestingly, the pool of Flk1+/Isl1+/Nkx2.5+ cardiac progenitors express multiple hematopoietic transcription factors. Interestingly, the pool of Flk1+/Isl1+/Nkx2.5+ cardiac progenitors express multiple hematopoietic transcription factors. Although a close relationship between cardiac, endocardial, and hematopoietic lineages has been suggested in Drosophila, zebrafish, and embryonic stem cell in vitro differentiation models, the activity of hematopoietic genes expressed in the developing mammalian heart remains unclear. Here, we examined the hemogenic activity of the

developing heart. Mouse heart explants from pre-circulation stages and Ncx1 mutant embryos generated myeloid and erythroid colonies in the absence of circulation. The hemogenic activity arose from a subset of endocardial cells in the outflow tract and atria earlier than in the aorta-gonad-mesonephros region. Furthermore, we found that a cardiac homeobox transcription factor, Nkx2-5, was expressed in and required for a hemogenic subset of the endocardium and yolk sac endothelium during embryogenesis. Together, these data suggest that Nkx2-5-dependent subset of endocardial and yolk sac endothelial cells serve as a de novo source for hematopoietic progenitors.

4.30 Characterization of the cardiac lymphatic system

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The lymphatic vasculature is a blind-ended network that is crucial for tissue fluid homeostasis, immune surveillance and lipid adsorption from the gut. The heart is the first, and conceivably most important, organ to develop in humans. However, the mechanisms regulating the development of the cardiac lymphatic vessels, as well as their cellular origin, are yet to be described. In this study, we shed light on the development and origin of the cardiac lymphatics from mid-gestation to early adulthood by utilizing immunohistochemistry and optical projection tomography (OPT). We show that lymphatic precursors are present from E14.5 in the epicardium, and vessels first sprout at E14.5 in the outflow region of the heart. Cardiac lymphatic vessels closely follow the path of coronary vessels as they develop, and by P15 the lymphatic vasculature encompasses the entire heart. Lineage tracing analysis suggests a non-endothelial origin for developmental de novo lymphvasculogenesis in the heart and indicates a putative involvement of the myeloid lineage. Our characterization of the cardiac lymphatics offers a novel insight into a largely overlooked, but arguably very important part of the lymphatic system. Future studies will focus on applying the findings from lymphatic development in the embryonic heart to adult cardiovascular pathology and disease.

4.31 Endothelin-1/Endothelin type-A receptor signaling regulates pharyngeal arch artery development through Dlx5/6-independent pathway.

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Endothelin-1 (Edn1) is a vasoconstrictor peptide, and is also involved in the development of cranial/ cardiac neural crest-derived tissues and organs. In craniofacial development, Edn1 binds to Endothelin type-A receptor (Ednra) to induce homeobox genes Dlx5/Dlx6 and determines the mandibular identity in the first pharyngeal arch. However, it remains unsolved whether this pathway is also involved in pharyngeal arch artery development to form the thoracic arteries. Here, we show that the abnormal branches from the common carotid arteries were observed in Edn1 or Ednra knock-out embryos, but not in Dlx5/Dlx6 double knock-out embryos. These anomalies derived from abnormal persistence of the first and second pharyngeal arch arteries. Abnormal smooth muscle cell differentiation from the neural crest cells was observed at the first and second pharyngeal arch arteries of Ednra knock-out embryos. These findings indicate that the formation of pharyngeal arch arteries does not depend o n Dlx5/Dlx6-mediated ventral identification of the pharyngeal arch, and the ET1/Ednra signal regulates the properly-directed differentiation of the neural crest cells into smooth muscle cells in the pharyngeal arch arteries.

4.32 Protection of oral hydrogen water as an antioxidant on pulmonary hypertension

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Background: This study was to explore protection of hydrogen as an antioxidant on monocrotaline (MCT)-induced pulmonary hypertension (PH). Instead of hydrogen-rich saline, we made new modification by using oral hydrogen water (HW), which is more convenient, less costly and longer time to be released. Methods: Thirty-six SD rats were equally randomized to three groups: SHAM group, MCT group and MCT+HW group. The PH model was established by intraperitoneal injection of MCT and the mean pulmonary arterial pressure (mPAP)

was measured two weeks later. MCT group were administered with ordinary drinking water, and MCT+HW group were administered with HW. Another two weeks later, the ventricular weight, left ventricular+septum weight, and right ventricle weight (RV) were measured. The right ventricular hypertrophy index (RVHI) was calculated. Damage to the lung tissue was observed and scored by HE staining. Changes of 3-nitrityrosine (3-nt) and Intercellular Adhesion Molecule-1 (ICAM -1) in the lung tissue were observed by immunohistochemistry. Results: mPAP, RV and RVHI in MCT group were significant higher than those in SHAM group ($P < 0.05$). mPAP, while RV and RVHI in MCT+HW group were significantly lower than those in MCT group ($P < 0.05$). In addition, 3-nt and ICAM-1 were increased significantly in MCT group and decreased significantly in MCT+HW group ($P < 0.05$). Conclusions: Oral hydrogen water has a significant protective effect on MCT-induced PH, which is more convenient to use than hydrogen-rich saline. And this effect is associated with the antioxidative ability of hydrogen and its action of reducing pulmonary inflammatory response.

4.33 Identification of a minimal arterial enhancer of Dll4 and implications for artery-vein specification.
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The transcriptional regulation of arteriovenous specification is not known. An essential role for VEGF and Notch signaling in establishing and maintaining vessel identity has been well-established. VEGF induces artery-specific expression of Dll4 and activates Notch signaling to promote arteriogenesis. In the mouse, Dll4 is the first arterial marker, and is required for arteriovenous specification. The transcriptional mediators that induce Dll4 expression remain unclear. To elucidate the transcriptional basis of artery-specific gene expression, we have employed transgenic reporter assays to identify the regulatory elements that drive artery-specific expression of Dll4 in mouse development. The promoter and upstream region of Dll4 is incapable of driving endothelial expression in mouse or zebrafish. We identified a minimal intronic enhancer of 30-bp that completely recapitulates the early and late developmental arterial-specific expression of Dll4. This activity is conserved in both mice and zebrafish. Mutagenesis of the Dll4 minimal enhancer indicates that Notch signaling via an RpbjK site is dispensable for arterial expression. The presence of other candidate transcription factor binding sites, including Ets sites, suggests potential regulatory mechanisms for arterial expression of Dll4. This enhancer does not recapitulate endogenous Dll4 expression in the post-natal mouse retina, suggesting that Dll4 expression may be regulated by different pathways during developmental vasculogenesis and post-natal angiogenesis. As we define the minimal enhancer of Dll4, we will be able to identify the transcriptional inputs required to induce artery-specific gene expression, and thus the minimal instructions required for arteriogenesis.

4.34 Transmembrane protein 2 (tmem2) is required for cardiovascular development
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The endocardium and blood vasculature are both derived from cells of the endothelial lineage and share common genetic regulators. *tmem2* was recently identified in zebrafish as a novel regulator of cardiac morphogenesis and we show here that *tmem2* is also required for angiogenesis. *tmem2* mutants are defective in intersegmental vessel sprouting for both primary (arterial) and secondary (venous) sprouting. In situ hybridization analysis of arterial and venous markers demonstrated that arterial-venous fate is correctly specified in *tmem2* mutants, supporting the notion that the angiogenesis defect is specific. Tmem2 is a 1390 amino acid protein with a single-pass transmembrane domain (located close to the N-terminus) and computer prediction places the large C-terminus in the extracellular space. Little else is known about Tmem2 structure or function. To investigate Tmem2 function, we have generated a novel transgenic tool to watch live protein dynamics in the developing embryo. Using BAC recombineering, we have generated a Tmem2-Cherry fusion protein under promoter control that is predicted to recapitulate endogenous expression. This transgene rescues the mutant phenotypes, demonstrating that we are observing the only functional copy of the protein as it performs its function. Live imaging of this line has provided novel insights into Tmem2 protein behavior and localization during angiogenic sprouting and cardiac morphogenesis. This work introduces a new regulator of angiogenesis and describes a novel tool that is providing unique insights into Tmem2 function.

4.35 Targeted non-invasive occlusion of pharyngeal arch arteries during avian cardiac morphogenesis

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The role of hemodynamics in outflow tract and pharyngeal arch artery morphogenesis is poorly understood. Through the use of two- photon microscopy guided femtosecond laser pulses, we nucleated and controlled the growth of microbubbles within outflow vessels without disturbing surrounding tissues. These bubbles temporarily occluded the vessel, during which time a stable occlusion could be formed by ablating the circulating thrombocytes that accumulated behind the bubble. These clot-like structures then persisted. Using this approach, we examine the effects of PAA vessel occlusions on embryonic viability, hemodynamic rearrangement, and downstream outflow tract morphogenesis. We determine that occlusion of the right IV pharyngeal arch artery caused lethality in 99.5% of embryos by day 6 (HH28/29), while occlusion of the left IV arch artery was significantly less lethal. In each case, blood flow was redistributed to the III and VI PAA and vessel diameters were altered significantly. With right IV PAA occlusion, the right III PAA artery increased 47% in diameter while the right VI decreased 100%. Interestingly, ultrasound derived blood velocity did not change in the right III PAA but significantly increased in the right VI. No changes in diameter or velocity were seen in the contralateral PAA. When both IV PAA were occluded, both PAA vessels changed in diameter, resulting in an overall decrease in ouflow lumen area. These results support the hypothesis that PAA hemodynamics is an important contributor to embryonic development.

4.36 Etv2 regulates endothelial development via activation of Sox7

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Etv2 has recently been shown to be an important factor for the specification of the endocardial/endothelial and hematopoietic lineages. Transgenic embryos lacking Et2v die between E9.0 and E9.5 due to absence of the endocardial/endothelial lineage and significant disruption of hematopoietic lineages. Sox 7, Sox 17 and Sox 18 comprise the Sox-F family which has been shown to be important in the formation of endoderm, regulation of hematopoietic cells and cardiovascular development. Given that the Etv2 null mice had defects in cardiovascular and hematopoietic development we questioned whether the Sox F members may be downstream targets of Etv2. Sox 7, Sox 17 and Sox 18 were found to be significantly downregulated in the Etv2 null mice compared to WT littermates at two timepoints (E8.0 and E 8.5). To mechanistically dissect the regulation of this molecular program, we utilized an array of molecular biological techniques to demonstrate that Etv2 is a direct upstream regulator of the Sox 7 gene. We generated a doxycycline-inducible Sox7-myc tagged overexpressing ES cell line. Overexpression of Sox7 in engineered EBs resulted in an induction of the endothelial pathway evidenced by an increase in the percentage of EB day 6, Flk-1/Cdh5 double positive endothelial progenitor cells and increased expression of Pecam1 and Cdh5 in protein harvested from Sox7 overexpressing EBs. Collectively, these studies support the conclusion that Etv2 is essential for the endocardial/endothelial lineage and Sox7 is an important factor in this developmental pathway.

Section 5: Coronaries, Epicardium, and Conduction System

S5.1 Ets-1 Regulates the Migration of Coronary Vascular Precurors and Coronary Endothelial Cell Proliferation

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We have recently described a cardiac developmental defect in mice deficient in the transcription factor Ets-1. These mice die in the perinatal period with ventricular septal defects and an ectopic focus of cartilage in their outflow tract myocardium. We demonstrated that these defects are the result of altered migration and differentiation of the cardiac neural crest due to the loss of Ets-1. In this report, we describe an additional role for Ets-1 during cardiac development. We observed left ventricular systolic dysfunction in Ets-$1^{-/-}$ mice as measured by a decrease in fractional shortening (45.3% vs. 22.5%, $p<0.0001$). In addition, we found a 36% reduction in myocardial capillary density in newborn Ets-1 deficient mice, consistent with the notion that the left ventricular dysfunction seen in Ets-$1^{-/-}$ mice is due to an inadequately developed myocardial capillary bed. To examine

earlier steps in coronary vasculogenesis, we performed whole-mount PCAM staining of E12.5 to E14.5 Ets-1$^{-/-}$ hearts and found significant attenuation in the development of the coronary vascular plexus. The appearance of both arterial and venous coronary precursors in the subepicardium was delayed, suggesting a global defect in the migration of these cells. In addition, phospho-histone H3 immunohistochemistry demonstrated a 41% reduction in the proliferation of endothelial cells in Ets-1$^{-/-}$ hearts at E16.5. Taken together, these results suggest that Ets-1 plays an important role in both the migration and proliferation of coronary endothelial cells during cardiac development.

S5.2 Wnt signaling in the developing murine epicardium and epicardium-derived-cells (EPDCs)

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The epicardium is the outermost layer of the heart and develops from a mesothelial cluster of cells at its venous pole (the proepicardium). A subset of these cells invades the underlying subepicardium and myocardium via an epithelial-to-mesenchymal-transition (EMT). Besides this cellular contribution additional epicardium-derived paracrine signals are important for myocardial and coronary vessel development. Previous work suggested a requirement of canonical Wnt signaling in the epicardium for EMT and coronary smooth muscle cell development. Here, we reinvestigated the role of canonical Wnt signaling in the epicardium and epicardium-derived-cells during embryogenesis, using an alternative conditional genetic approach. In the background of a Tbx18cre-line showing recombination in the proepicardium and its derivatives, ß-catenin (Ctnnb1) loss- and gain-of-function alleles were analyzed. Surprisingly, mice deficient for Ctnnb1 are live born and show neither impaired coronary artery formation, nor a defective epicardium. The ability of epicardium-derived explants to differentiate into the smooth muscle lineage is maintained. Stabilization of Ctnnb1 in the epicardium resulted in lethality between embryonic days eleven and twelve. Further lineage analysis revealed the formation of undifferentiated cell aggregates on the surface of the developing heart and impaired EMT. Our data contradicts previous work, by demonstrating that canonical Wnt signaling in epicardium and EPDCs is dispensable for coronary artery formation in the developing embryo. Moreover constitutive active Wnt signaling in these cells leads to early lethality, indicating only a minor physiological role of Wnt signaling in the epicardium and epicarium-derived-cells during development.

S5.3 Determination of Cardiac Pacemaker Cell Origins Reveals a Critical Role for Wnt Signaling During their Cell Fate Specification.

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Cardiac pacemaker cells have unique molecular and physiological characteristics that allow them to rapidly and autonomously generate action potentials. How and when pacemaker cell fate diversifies from adjacent myocardial cell types and are programmed to attain these specialized features, however, remains poorly understood. Here we report our fate mapping studies demonstrating that just following gastrulation pacemaker progenitors reside in the right lateral plate mesoderm posterior to the known heart fields. Our data further indicate that this pacemaker precursor region overlaps with the expression of at least one canonical Wnt, Wnt8c. This is surprising as canonical Wnts are thought to be inhibitory for myocardial specification during these stages. In order to test whether Wnt signaling is required to specifically induce pacemaker versus working myocardial fate, we injected cells expressing the soluble Wnt antagonists, Crescent, directly into the pacemaker region of gast rulating embryos. Ectopic Crescent resulted in both the expansion of the transcription factor NKX2.5 and the down-regulation of at least one pacemaker ion channel, HCN4, within the pacemaker precursors. Conversely, introduction of canonical Wnts into the heart field mesoderm was capable of inducing ectopic pacing sites and the formation of NKX2.5 negative HCN4 positive myocytes within the looping heart tube, suggesting a conversion of working myocytes into pacemaker-like cells. These data demonstrate that pacemaker progenitor specification depend on signaling cues unique from working myocardium and that Wnt signaling, at least in part, supports divergence into the pacemaker lineage. This work is supported in part by grants from the NIH-NLHBI.

S5.4 A Tbx5-Scn5a Molecular Network Modulates Function of the Cardiac Conduction System

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Cardiac conduction system (CCS) disease is common with significant morbidity and mortality. Current treatment options are limited and rational efforts to develop cell-based and regenerative therapies require knowledge of the molecular networks that establish and maintain CCS function. Emerging evidence points to key CCS roles for genes with established roles in heart development. Recent genome wide association studies (GWAS) have identified numerous loci associated with human CCS function including TBX5 and SCN5A. We hypothesized that Tbx5, a critical developmental transcription factor, regulates networks required for mature CCS function. Removal of Tbx5 from the mature VCS resulted in severe VCS functional consequences, including loss of fast conduction, arrhythmias, and sudden death. Ventricular contractile function and the VCS fate map remained unchanged in VCS-specific Tbx5 knockouts. However, key mediators of fast conduction including Cx40 and Nav1.5, encoded by Scn5a, demonstrated Tbx5-dependent expression in the VCS. We identified a Tbx5-responsive enhancer downstream of Scn5a sufficient to drive VCS expression in vivo, dependent on canonical T-box binding sites. Our results establish a direct molecular link between Tbx5 and Scn5a and establish a hierarchy between human GWAS loci that affects VCS function in the mature CCS, establishing a paradigm for understanding the molecular pathology of VCS disease. Ongoing studies to dissect the regulation of Scn5a expression using a Scn5a-LacZ BAC transgenic reporter system have identified two Scn5 a enhancers sufficient for establishing the native Scn5a expression pattern. Implications for the role of Scn5a and Scn10a in conduction system function, variability, and arrhythmia susceptibility will be discussed.

S5.5 Mouse cardiac T-box targets reveal an Scn5a/10a enhancer functionally affected by genetic variation in humans

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The contraction pattern of the heart relies on the activation and conduction of the electrical impulse. Perturbations of cardiac conduction have been associated with congenital and acquired arrhythmias and cardiac arrest. The pattern of conduction depends on the regulation of heterogeneous gene expression by key transcription factors and transcriptional enhancers. Here, we assessed the genome-wide occupation of conduction system regulating transcription factors Tbx3, Nkx2-5 and Gata4 and of enhancer-associated co-activator p300 in the mouse heart, uncovering cardiac enhancers throughout the genome. Many of the enhancers co-localize with ion channel genes repressed by Tbx3, including the clustered sodium channel genes Scn5a, essential for cardiac function, and Scn10a. We identify two enhancers in the Scn5a/Scn10a locus, which are regulated by Tbx3 and its family member and activator Tbx5, and are functionally conserved in humans. We provide evidence that a single-nucleotide po lymorphism in the SCN10A enhancer, associated with alterations in cardiac conduction patterns in humans, disrupts Tbx3/5 binding and reduces the cardiac activity of the enhancer in vivo. Thus, the identification of key regulatory elements for cardiac conduction helps to explain how genetic variants in non-coding regulatory DNA sequences influence the regulation of cardiac conduction and the predisposition for cardiac arrhythmias.

S5.6 Activation of voltage-dependent anion channel 2 suppresses Ca^{2+}-induced cardiac arrhythmia

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While a role of Ca^{2+} in establishing embryonic cardiac rhythmicity has been proposed, the underlying mechanisms have yet to be fully explored. The zebrafish *tremblor* (*tre*) mutant embryo lacks functional cardiac Na^{+}/Ca^{2+} exchanger, resulting in Ca^{2+} extrusion defects, abnormal Ca^{2+} transients and unsynchronized cardiac contractions. We therefore use this mutant as an animal model to dissect the molecular network essential for controlling Ca^{2+} homeostasis in the heart. From a chemical suppressor screen, we identified a synthetic compound named efsevin, which effectively restores synchronized cardiac contractions in *tre* embryos. In addition, efsevin suppresses arrhythmogenic Ca^{2+} waves by accelerating the decay phase of Ca^{2+} sparks in

murine cardiomyocytes, demonstrating that efsevin modulates cardiac Ca^{2+} handling. Through a biochemical pull-down assay, we identified a direct interaction between efsevin and the mitochondrial outer membrane protein VDAC2. Overexpression of VDAC2 restores synchronized cardiac contractions in *tre* embryos and knock-down of VDAC2 attenuates the rescue effect of efsevin, indicating that efsevin modulates Ca^{2+} handling by enhancing the activity of VDAC2. Furthermore, overexpression of mitochondrial inner membrane Ca^{2+} uptake proteins restores cardiac contractions in *tre* embryos. This effect is further enhanced when VDAC2 is co-expressed, and is abolished in *tre/vdac2* double deficient embryos. Taken together, our data reveal an essential role for mitochondria in regulating cardiac Ca^{2+} homeostasis and rhythmicity. Our findings also establish VDAC2 as a critical gate for mitochondrial Ca^{2+} uptake and efsevin as a potential therapeutic agent for cardiac arrhythmia.

5.7 The BMP regulator BMPER is necessary for normal coronary artery formation

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Connection of the coronary vasculature to the aorta is one of the last essential steps of cardiac development. However, little is known about the signaling events that promote normal coronary artery formation. The bone morphogenetic protein (BMP) signaling pathway regulates multiple aspects of endothelial cell biology but has not been specifically implicated in coronary vascular development. BMP signaling is tightly regulated by numerous factors, including BMP-binding endothelial cell precursor-derived regulator (BMPER), which can both promote and repress BMP signaling activity. Analysis of the BMPER-/- mouse indicated that this deficiency leads to embryonic death, and in the embryonic heart, BMPER expression is limited to the endothelial cells and the endothelial-derived cushions, suggesting that coronary vascular defects may be present. Histological analysis of BMPER-/- embryos at embryonic day 16.5 revealed that the coronary arteries were either atretic or connected distal to the semilunar valves. However, analysis of earlier embryonic stages showed that the coronary plexus began differentiating normally and that apoptosis and cell proliferation were unaffected in BMPER-/- embryos. In vitro tubulogenesis assays showed that isolated BMPER-/- endothelial cells had impaired tube formation compared to wild-type endothelial cells. Together, these results indicate that BMPER-regulated BMP signaling is critical for the migration of coronary endothelial cells and normal coronary artery development.

5.8 Epicardial chemokine signaling influences ventricular wall proliferation and coronary vasculogenesis

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During heart development, the epicardium secretes soluble factors that direct the morphological organization of the underlying myocardial wall. Our previous study established that insulin-like growth factor 2 (IGF2) is the primary mitogen expressed by the epicardium that controls embryonic ventricular cardiomyocyte proliferation. We found that CXCL12 (stromal derived factor-1) is an additional factor expressed by epicardial cells in vitro and by the epicardium in vivo. Cultured primary cardiomyocytes treated with epicardial conditioned media or in coculture with epicardial cells show a proliferative response that is partially blocked by AG1024 (an IGFR inhibitor) or AMD3100 (a CXCR4 inhibitor), and almost completely abolished by both compounds together. To date, a functional role for CXCL12 signaling in heart development has not been examined. Our results indicate that both global Cxcl12 null and cardiac-specific (Nkx2.5Cre) Cxcr4 null embryos show a moderate deficiency in compact zone proliferation. Embryos with combined loss of IGF2 and CXCL12 signaling exhibit pronounced edema, suggesting that epicardial IGF2 and CXCL12 together promote mitogenic signaling needed for cardiomyocyte proliferation and compact zone expansion. Because CXCL12 is a chemokine that has known effects on vascular cells, we also examined coronary vessel formation in embryos with disrupted CXCL12 signaling. We observed expanded superficial vessels and a reduced number of intramyocardial arteries. Our results indicate that the epicardium is a source of secreted CXCL12 that participates with the major mitogen IGF2 to induce cardiomyocyte proliferation and compact zone expansion, and also acts on endothelial cells to support formation of the coronary vasculature.

5.9 The role of Pod1/Tcf21 in epicardium-derived cells during cardiac development and fibrosis

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During cardiac development, epicardium-derived cells (EPDCs) differentiate into fibroblasts and vascular smooth muscle (SM) cells. In the adult heart, EPDCs are reactivated upon cardiac injury. Immunofluorescence studies demonstrate that the transcription factors (TF) Pod1/Tcf21, WT1, Tbx18, and NFATC1 are expressed heterogeneously in EPDCs in chicken and mouse embryonic hearts. Expression of Pod1 and WT1, but not Tbx18 or NFATC1, is activated with all-trans-retinoic acid (RA) treatment of isolated avian EPDCs. In intact E7 chicken hearts, RA signaling is required for full Pod1 expression and RA treatment inhibits SM differentiation. The requirements for Pod1 in differentiation of EPDCs in the developing heart were examined in mice lacking Pod1. Loss of Pod1 in mice leads to epicardial blistering, increased SM differentiation on the surface of the heart, and a paucity of interstitial fibroblasts, with neonatal lethality. On the surface of the myocardium, expression of multiple SM markers is increased in Pod1-deficient EPDCs, demonstrating premature SM differentiation. Together, these data demonstrate a critical role for Pod1 in controlling EPDC differentiation into SM during cardiac development. In adult hearts, cardiac injury leads to reactivation of fetal genes including WT1, Tbx18, and RALDH2 in EPDCs. Initial studies demonstrate that Pod1/Tcf21 expression is increased in mouse hearts subjected to myocardial infarction, transverse aortic constriction, or chronic isoproterenol infusion. Additional data will be presented describing Pod1/Tcf21 expression in mouse models of cardiac fibrosis. Together this work provides evidence for conserved Pod1/Tcf21-related regulatory mechanisms in EPDCs during cardiac development and fibrotic disease.

5.10 Epicardial GATA factors regulate coronary endothelial migration via Sonic hedgehog signaling.

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Early coronary vasculogenesis is dependent on the epicardium as a source of progenitor cells and regulatory signals. GATA transcription factors have been shown to be required for the formation of the proepicardium, a group of cells that give rise to the epicardium This suggests GATA factors may play a role in epicardial function. To address whether epicardial GATA-4 and GATA-6 may have a role in early coronary vascular development, we utilized a Wilm's Tumor-1 promoter-driven Cre recombinase construct to conditionally knockout GATA-4 and GATA-6 in the epicardium. We found the combined loss of GATA-4 and GATA-6 resulted in embryonic lethality at E15.5, an age at which various coronary vascular defects commonly lead to lethality. Immunofluorescent imaging at E13.5 and E14.5 indicated an 80-90% decrease in sub-epicardial endothelial cells, which are required for coronary plexus formation. This led us to hypothesize that the decreased number of sub-epicardial endothelial cells is due to a loss of epicardial signaling to early endothelial or progenitor cells. Sonic hedgehog is an epicardial signaling factor required for the migration of sub-epicardial endothelial cells. Therefore, we immunofluorescently stained for Sonic hedgehog and found its expression was lost in the sub-epicardial space of the conditional knockout. These results are consistent with a novel role for epicardial GATA factors in coronary vascular development through regulation of a Sonic hedgehog signaling pathway

5.11 Coordinated Regulation of Pacemaker Ion Channels during SAN Differentiation

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Cardiac pacemaker cells (CPCs) rhythmically generate electrical signals that dictate the timing of heart contractions. While the ion currents responsible for pacemaker action potentials (APs) have been thoroughly investigated, the developmental mechanisms regulating the underlying channels and pumps remain poorly understood. We have previously reported that CPC fate is specified in the mesoderm posterior to the heart field 2 full days before they first become electrically active within the right inflow. To determine the underlying mechanisms for this lag between fate specification and differentiation, we monitored the expression of ion handling genes necessary for CPC APs during this developmental window. Here we report that HCN4, Serca2, NCX, and RYR2 are all absent from CPC precursors, but then become up-regulated broadly across the right inflow as the CPCs first begin pacing the heart. To determine if this coordinated upregulation is dependent on known CPC transcriptional programs, we examined the expression of T-box genes Tbx3, Tbx5, and Tbx18. Although detectable in the inflow, their expression patterns did not match that of the CPC ion channels nor were any expression profiles confined to the pacemaking site. This data suggest that the molecular components

necessary for CPC AP generation are regulated in a coordinated manner and their upregulation dictates the timing of CPC electrical activation. Additionally, the site of action potential production cannot be demarcated solely by the above factors, indicating that further transcriptional and/or signaling mechanism(s) may be required. Supported by NIHR01HL112268.

5.12 Cardiac Specific Expression of FOG-2 Induces Atrial Fibrillation

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Atrial fibrillation is a common cardiac arrhythmia associated with significant worldwide morbidity and mortality. Although many factors, including myocyte electrical remodeling, atrial contractile dysfunction, and structural remodeling have been linked to atrial fibrillation, the molecular causes are still unclear. Previous work has demonstrated that FOG-2 (Friend of GATA-2), a transcriptional co-repressor important in early heart development, is overexpressed in the hearts of patients with heart failure who are prone to developing atrial fibrillation. We have developed a novel genetic model of inducible, cardiac specific FOG-2 expression in mice via a doxycycline-inducible transgenic system. In these mice, we find an approximately 5-7 fold increase in FOG-2 protein and mRNA levels in the atrial tissue of these animals following gene induction. These animals have a high incidence of spontaneous and sustained atrial fibrillation (approximately 80%), with the remainder of these animals having easily induced atrial fibrillation by catheter-based pacing of the right atrium. The atrial fibrillation precedes any left ventricular systolic dysfunction, valvular disease, or elevated preload, indicating a primary atrial mechanism. Because FOG-2 is known to inhibit the expression of multiple cardiac-specific genes, we performed a microarray-based gene expression analysis on atrial tissue. The results of this microarray suggested that the expression of multiple potassium channel subunit proteins (KCNJ5, KCND2 and KCNE1) are downregulated, and these results were further confirmed by quantitative RT-PCR. These data suggests that FOG-2 upregulation leads to the downregulation of cardiac potassium channels, thus promoting the development of atrial fibrillation.

5.13 Localization and lineage analysis of the sinoatrial node precursor cells within the posterior Secondary Heart Field in the mouse

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The early heart forms from two mesodermal cell populations, the First and Second Heart Fields (F&SHF). The FHF forms the cardiac crescent (E7.5 in mouse). The SHF lies dorsally and medially, in pharyngeal mesoderm, and is defined by *islet1* expression. The left ventricle (LV) derives exclusively from FHF whereas the distal outflow tract (OFT) is SHF derived. The proximal OFT and RV are predominantly SHF derived, and the atria are of mixed F&SHF origin. A sub-portion of the SHF expresses *Fgf10*, as shown by the 1V-24 lacZ reporter, which labels the anterior HF (AHF). The AHF is fated to contribute to the OFT/RV but not the inflow/atria. Using dye-labeling injections at the posterior portion of the SHF (pSHF), which is characterized by Islet1+/1v-24- expression, we have demonstrated that IF region arise from pSHF (see Galli et al, 2008) and also atrioventricular canal (AVC) and OFT are pSHF-derived cardiac regions (Domínguez et al, 2012; submitted). Lineage studies using islet1-cre and islet1-mer-cre-mer mice crossed with the ROSA26R, have described that sinoatrial node (SAN) and a central region of the atrioventricular node (AVN) are derived from islet1 precursor cells (SHF) (Moretti et al, 2006; Sun et al, 2007), whereas it is assume that ventricular conduction system (His-Purkinje system) is a non-SHF derived. In this regard, an accurate lineage analysis of SHF-islet1 cells contribution to the distinct components of the cardiac conduction system (CCS) is missing. Moreover, the localization of precursor cells forming the SAN and AVN central region from the SHF is unknown. Base on our previous data, we are interesting now to determine whether SAN and AVN islet1+ precursor cells are placed at the pSHF and, moreover, whether islet1+ progenitor cells participate in His-Purkinje system development during cardiogenesis. We are labelling small groups of cells of 4-6 somites mouse embryos, throughout the pSHF using fluorescent dyes. After that, we culture the embryos for 48 hours and then we perform immunohistochemistry in whole mount against HCN4, a well-known SAN molecular marker. Preliminary results evidence that, in most cases, right and left pSHF derived cells populate the right and left atria of the developing heart (HCN4-), respectively. However, some dye-labelled cells were placed at the right atria-sinus venous junction, where HCN4 is expressed and define the putative SAN precursor. These results would suggest that islet1+ progenitors cells localized at the pSHF participate in the developing of both, the working and conduction

system myocardium, and open new ways to explore the putative SHF origin of the distinct cardiac conduction system elements.

5.14 Emergence of novel myocardial subtypes during dynamic patterning of impulse conduction pathways at the pacemaker-atrial junction

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Cardiac pacemaker cells of the sinoatrial node initiate electrical impulses that drive rhythmic contraction of the heart. In the mature heart, pacemaker cells are protected from electrical suppression imposed by adjacent working myocardium by a zone exhibiting poor electrical coupling. However, little is known about the developmental mechanisms that pattern the low conductance zone that is critical for proper pacemaker functions. Here we report that pacemaker precursors differentiate within the ventral surface of the right inflow during heart looping and begin to produce pacemaker action potentials (AP). At this stage, APs propagate along the ventral surface directly into the forming atria and no low conductance zone is evident at or around the pacemaker cells. As septation initiates, a dynamic shift in propagation pattern occurs as a conduction delay forms at the junction of the right inflow and the atria, and a novel dorsal propagation pathway becomes the dominant route. Importantly, the cells that compose this zone uniquely express slow skeletal myosin heavy chain. These junctional myocytes are distinguishable from pacemaker cells that co-express Ventricular myosin heavy chain1 (VMHC1) and Atrial myosin heavy chain 1(AMHC1), and atrial myocytes that express AHMC1 alone. These data demonstrate dynamic patterning of conduction properties at the pacemaker-atrial junction and suggest that these properties may be related to the emergence of myocyte subtypes with distinct transcriptional programs. Supported by R01 HL093566.

5.15 Endocardium-derived Follistatin-like-1 determines atrioventricular conduction.

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In the adult heart the atrioventricular (AV) node generates the delay between atrial and ventricular activation. During development the AV delay is generated by the slowly conducting AV canal myocardium, from which the demarcated AV node forms. How the AV canal myocardium becomes (1) confined to an AV-node and (2) insulated by connective tissue, is largely unknown. Follistain-like 1 (Fstl1) is a secreted protein that potentially regulates cardiac development by binding TGFβ super-family members, like BMPs. Fstl1 is expressed from gastrulation onwards in various organs including the heart, particularly in the endocardially-derived cells that contribute, to the developing AV valves and insulating plane. Inactivation of Fstl1 in mouse results in post-natal death due to respiratory defects. To determine the role of endocardial-derived Fstl1 in the formation of the AV node, we generated endocardium-specific Fstl1 KO mice and studied the structure and function of the AV junction. Endocardium-specific, but not myocardium-specific, deletion of Fstl1 resulted in PR prolongation in neonates, underscoring the specific role of endocardium-secreted Fstl1 in AV node function. Within four weeks after birth these mice continued to have a prolonged PR interval and died. In these mice, primitive AV canal myocardium, marked by Hcn4, was still present below the valves. BMP reporter assays revealed that Fstl1 is capable to inhibit BMP signalling, albeit only at low concentrations. Taken together our data suggests that endocardium-derived Fstl1 inhibits BMP at low concentration, which is required for normal development of the AV junction including the AV node.

5.16 Assessment of the genome-wide occupation of cardiac transcription factors reveals two conserved T-box factor-regulated enhancers for the expression patterns of *Scn5a* and *Scn10a*

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Repression of the working myocardial phenotype is essential for the development of the nodal components of the conduction system. Tbx3 plays a central role in this process, regulating the expression of many key

conduction genes and thereby defining the electrophysiological phenotype of the sinus and atrioventricular nodes. The specific down-regulation of connexins and ion channels that are associated with rapid conduction is essential for the correct delay and thus function of the atrioventricular node. The cardiac sodium channel protein, Scn5a, is an essential mediator of cardiomyocyte polarization and plays a central role in rapid conduction of the electrical impulse. Mutations in Scn5a give rise to a wide spectrum of conduction defects and have been associated with an increased risk of ventricular fibrillation. Loss of Tbx3 expression results in the ectopic expression of Scn5a in the nodal components of the conduction system and over-expression of Tbx3 in working myocardium reveals its down-regulation. Using a genome wide ChIP-seq approach we have identified functional binding sites for Tbx3 and cardiac partners of Tbx3, Gata4 and Nkx2-5, across the mouse genome. Combining this data with other ChIP-seq data and microarray data we identified a locus encompassing both *Scn5a* and *Scn10a*, possessing 2 putative enhancers. *In vivo* enhancer screening confirmed the ability of these DNA elements to drive LacZ expression in a similar patterns to that of endogenous Scn5a/10a and that these elements are conserved in humans. We show that a recently identified GWAS SNP connected to alterations in AV-conduction and positioned directly under a ChIP-seq peak of one of the enhancers, appears to attenuate T-box binding to this enhancer, thus interfering with transcription regulated by this enhancer. The effect of this attenuation is a loss of correct ventricular expression, an assessment based on *in vivo* analyses of the human enhancer in both fish and mouse model systems.

5.17 Exclusion of Endothelin Signaling is Required for Slow Conducting Fate in the Atrioventricular Junction

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Efficient blood flow requires sequential and unidirectional contraction of cardiac chambers. The Atrioventricular junction (AVJ) delays action potential propagation between the developing atria and ventricles allowing for atrial contraction to complete before ventricular activation. This delay represents the first detectable heterogeneity in conduction velocity in the heart. The mechanisms responsible for establishing this heterogeneity, however, are poorly understood. Previously our group has demonstrated that in the embryonic chick ventricle, endothelin is capable of inducing the expression of a high conductance gap junction connexin 40 (Cx40) which is present in the fast conducting Purkinje fiber network but absent from the AVJ. We therefore sought to address whether lack of endothelin signaling in the AVJ is required for this region to escape a fast conducting fate. Here we report that while one of the ET receptors, ETB, is excluded from the developing AVJ, all oth er Endothelin signaling components, including ppET1, ECE1 and ETA are expressed there. Furthermore, the receptor ETA can function in the AVJ as exogenous endothelin ectopically induced both the phosphorylation of a downstream endothelin signaling component, Erk, and Cx40 expression at the AVJ. These results demonstrate that AVJ cells can respond to endothelin signaling converting them from slow conducting cells into fast conducting cells as in the ventricles. The data also suggest that there must be a mechanism that excludes endothelin signaling from this region thereby allowing AVJ cells to escape the fast conducting cell fate. Supported by NIH-R01 HL093566, Cardiovascular Diversity Research Supplement, and CIRM.

5.18 Pitx2 prevents the onset of an arrhythmogenic program in the mediastinal myocardium: implications for adult heart function

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Genome wide study populations in humans have indicated PITX2 as a candidate susceptibility gene for atrial arrhythmias, also confirmed by functional studies in adult Pitx2 heterozygous mice. Arrhythmogenic foci mostly originate in the pulmonary veins (PV) or in left sinus venosus (SV) derived structures, such as the coronary sinus or the left superior caval vein; the posterior left atrial wall is additionally critical both for initiation and maintenance of atrial fibrillation. We have previously shown that Pitx2 is required in the SV myocardium to progressively confine its molecular and functional pacemaker properties exclusively to the right SAN. We now present the role of Pitx2 in the mediastinal myocardium, a Cx40 positive/ANF negative region comprising the PV myocardium, the primary and secondary interatrial atrial septum (IAS) and left dorsal atrial wall. Pitx2 is expressed in the left mediastinal region and the derived mediastinal myocardium. We show here that deletion of Pitx2 from cardiomyogenesis onset leads to a reduced extension of the PV myocardium and to its complete reprogramming towards a nodal-conductive type molecular profile. Additionally, in the Pitx2 myocardial mutants the dorsal atrial wall and the IAS express ANF, which is molecular substrate for AF.

Altogether, the nature of Pitx2 molecular targets and their topological localization provide support for an anti-arrhytmogenic role of the gene at the left venous pole of the heart.

5.19 Islet-1 expression identifies the sinoatrial pacemaker of the adult zebrafish heart

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Cardiac development and function are strikingly conserved amongst animal classes. Although the heart's shape, relative size and organization may vary, its rhythmic contractions remain crucial for correct cardiac function. In higher vertebrates such as mammals, specialized pacemaker cells controlling the rhythmicity of cardiac contractions are localized in an anatomically identifiable structure of myocardial origin, the sinoatrial node (SAN). The SAN's anatomical and functional identification dates back to the early 20th century, before the availability of any molecular marker. In lower vertebrates, however, although cardiac contractions are known to originate at the venous pole, a localization that is developmentally conserved, isolation of such structure has remained elusive. Here we show that zebrafish embryos lacking the LIM/homeodomain-containing transcription factor Islet-1 (Isl1) display cardiac rhythmicity defects related to pacemaker dysfunction. Moreover, 3D reconstructions of gene expression patterns in the embryonic and adult zebrafish heart enabled us to uncover a previously unidentified, ring-shaped region of interconnected isl1+ cells in the hcn4+ tbx2b+ nppa- myocardium at the sinoatrial junction. Using a tg(Isl1BAC:GalFF; UAS:GFP) reporter line made in our laboratory, we demonstrated by optical mapping of action potentials and electrophysiological characterization of single isl1+ myocytes that the isl1-expressing cells harbor pacemaker activity. Altogether, our data allow us to establish that (i) isl1 is the first identified molecular marker for pacemaker function of the zebrafish heart and (ii) the functional pacemaker of the zebrafish is organized as a ring at the sinoatrial junction.

5.20 Phenotypic analysis of the cardiac conduction system in the absence of *Tbx1*

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The ventricular conduction system (VCS) is responsible for the rapid propagation of electrical activity in the ventricles in order to synchronize cardiac contraction. The VCS is composed of specialized cardiomyocytes forming the electrical wiring of the ventricles including the His bundle, the right (RBB) and left (LBB) bundle branches and the peripheral Purkinje fibers (PF). *TBX1* is a major candidate gene for DiGeorge syndrome, characterized by craniofacial defects and cardiac malformations. Several cardiac defects mimicking the human syndrome can be observed in *Tbx1* null mouse embryos such as common arterial trunk and ventricular septal defect. At E18.5, *Tbx1* null hearts present a distinct phenotype with a non-compact His bundle and absence of proximal RBB. Electrical activation maps of *Tbx1* mutant hearts revealed a left activation of ventricles while two breakthroughs in the right and left ventricles are observed in control hearts. This study revealed that the *Tbx1* mutants display a RBB block. At E14.5, the His bundle is positioned more anteriorly in *Tbx1*$^{-/-}$ hearts but no significative differences were observed by optical mapping in *these* hearts in accordance with the important network of trabeculae present in the right ventricle. This is in accordance with the incomplete insulation between atria and ventricule at this stage and the presence of embryonic electrical pathway at the atrioventricular junction. Altogether, these data demonstrate that the absence of the RBB underlie a RBB block. This phenotype may occur during fetal stages. To conclude, our data strongly suggest that a defect in cardiac morphogenesis may be at the origin of conduction defect suggesting that a correct development of the VCS is important to acquire a functional heart.

5.21 Epicardial Cells lacking *Tgfbr3* have Dysregulated NFkB Signaling

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The epicardium plays an important role in coronary vessel formation. Identifying the genes and signaling pathways which regulate epicardial cell behavior during development may provide key insights not only into coronary vessel development but also epicardial-mediated cardiac repair in adults. *Tgfbr3*$^{-/-}$ mice exhibit failed coronary vessel development associated with decreased epicardial cell invasion and proliferation, yet the exact mechanism is unknown. *Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$ epicardial cells were incubated with ligands that drive invasion via TGFbR3, TGFb1 [250 pM], TGFb2 [250 pM], or BMP2 [5000 pM], to identify genes dysregulated after receptor loss. 72 hours after ligand addition cells were harvested for RNA sequencing analysis. 10 million reads identified ~14,000 significantly expressed genes per condition. Several genes were found to be 2-fold differentially expressed between *Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$ cells ($p<0.001$) when incubated with vehicle (812), TGFb1 (936), TGFb2 (962), or BMP2 (673). Gene Regulatory Networks constructed from these gene lists identified NFkB as a key nodal point across all ligands examined. *Tgfbr3*$^{-/-}$ cells exhibited decreased expression of 32 genes known to be activated by NFkB signaling. NFkB activity was higher in *Tgfbr3*$^{+/+}$ cells after TGFb2 or BMP2 incubation. *Tgfbr3*$^{-/-}$ cells failed to activate NFkB in response to these ligands. Immortalized epicardial cells incubated with an inhibitor of NFkB activity no longer invaded into a collagen gel in response to TGFb1, TGFb2, or BMP2. Together, these data suggest that NFKb signaling is dysregulated in *Tgfbr3*$^{-/-}$ epicardial cells and that NFkB signaling is required for epicardial cell invasion *in vitro*.

5.22 T-box transcription factor 18 is dispensible for epicardial development, but repressive T-box protein function may be involved

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Tbx18, a member of the T-box family of transcription factors, is expressed in two areas of the splanchnic mesoderm caudal-laterally from the heart tube at E8.25 of murine development. These areas fuse in the septum transversum and give rise to the proepicardium (PE) at E9.25. Later, Tbx18-positive cells from the PE delaminate and populate the surface of the growing heart tube as a mesothelium called epicardium, which is a source of coronary smooth muscle cells and cardiac fibroblasts. Shortly before birth, the expression of Tbx18 in the epicardium is lost indicating the end of cell differentiation from this outermost layer of the heart. Here, we analyse the function of Tbx18 in the development of the epicardium and its derivates. Using Tbx18 null and Tbx18 overexpressing mice we could show that the loss as well as the gain of Tbx18 did not interfere with epicardial development or the fate of epicardium-derived cells. Nevertheless, additional studies of mice overexpressing an activating form of Tbx18 (epiTbx18VP16) prematurely differentiate into smooth muscle cells. Inhibition of Tgfb- and Notch-signalling in epiTbx18VP16 epicardial explants prevented the differentiation into smooth muscle cells. We suggest that repressive T-box protein function may be necessary for epicardial development by inhibition of Tgfb- and Notch-signalling in the embryonic epicardium.

5.23 Epicardial HIF1 regulates vascular precursor cell invasion into the myocardium through the VEGF signaling pathway

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Our previous data suggested a link of epicardial HIF and coronary vasculogenesis. To better understand the autocrine role of HIF during this process, we transduced adenovirus mediated expression of constitutively active HIF1α in the embryonic avian epicardium. We observed over-expression of HIF1α in the epicardium prevented epicardial derived cells (EPDC) from proper migration into the myocardium. In vitro collagen gel assays and ex ovo heart culture further confirmed that infection with AdcaHIF1α impaired the ability of epicardial cells to invade. Results from immunostaining and real-time PCR suggested that epicardial EMT and EPDC differentiation into endothelial cells and smooth muscle cells was not disrupted. Further investigation revealed the transcript level of Flt-1, which can act as a VEGF signaling inhibitor, increased several fold after introducing AdcaHIF1α. Moreover, blocking the activation of the VEGF pathway in epicardial cells recapitula ted the inhibition of EPDC invasion. These results suggest that caHIF1α mediated up-regulation of Flt-1 blocking the activation of the VEGF pathway is responsible for the inhibition of EPDC myocardial migration. In conclusion, our studies support that epicardial HIF is required for regulating epicardially derived mesenchyme invasion into the myocardium, a critical step in the vascularization of the myocardium. This role of HIF indicates that the differentially hypoxic microenvironment may inform epicardial cells when to stop as well as when to start migration. This work was supported by American Recovery and Reinvestment Act (ARRA) funds through grant number R01HL091171 to M.W. and R01HL96597 to D.R-B.

5.24 Dioxin Exposure Impairs Epicardium Development in the Zebrafish and Mouse

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The vertebrate primordial heart is comprised of the myocardium and endocardium. Further development of the heart relies heavily on the formation of the epicardium, which is the outer-most layer of the heart. The epicardium is derived from a transient extra-cardiac mesothelial organ called the proepicardium (PE). In zebrafish (*Danio rerio*), exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin, TCDD) at 24 hours post fertilization blocks formation of the epicardium. We hypothesized that failure to form the epicardium might be secondary to a TCDD-induced disruption of PE formation. To test this hypothesis, we scored for the presence or absence of the PE in zebrafish embryos exposed to TCDD. From our experiments it is clear that TCDD-exposed zebrafish fail to form a PE. Furthermore, *in situ* hybridization and immunohistochemistry revealed that transcription factor 21 (*tcf21*) expression was absent at the PE site following TCDD exposure. Cardiac malformations caused by TCDD exposure are not limited to zebrafish. Embryonic chick (*Gallus gallus domesticus*) and mouse (*Mus musculus*) hearts are also targets of TCDD toxicity; therefore, we broadened our investigation and hypothesized that TCDD causes PE or epicardial defects in these other vertebrates. TCDD exposure did not inhibit formation of the PE or epicardium in the chick and mouse. However, the TCDD-exposed mouse epicardium was distended from the underlying myocardium at E12. Supported by NIH Grant ES012716 and UW Sea Grant.

5.25 Rescue of Coronary Development by PDGF-BB and Sonic Hedgehog in FOG-2 Deficient Hearts

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Homozygous targeted mutation of the transcriptional co-repressor FOG-2 in mice results in mid-gestational embryonic death due to profound structural abnormalities in the heart. Interestingly, FOG-2 deficient animals form an intact epicardium, but fail to elaborate a coronary vascular plexus by embryonic day (e) 12.5. In contrast, wild-type littermates have a well-developed coronary plexus by e12.5, suggesting that the loss of FOG-2 prevents coronary plexus formation. Taking a candidate gene approach, we identified two genes, PDGF-B and Sonic Hedgehog (Shh), that are downregulated in the hearts of FOG-2 deficient animals. We observe that PDGF-BB and Shh protein can each rescue coronary plexus formation in mutant hearts grown *in vitro*. Further we demonstrate that cyclopamine, a potent inhibitor of Shh signaling, prevents coronary plexus rescue by PDGF-BB, suggesting that PDGF-BB works upstream of Shh. Collectively our observations support a model where FOG-2 supports coronary vasculogenesis through upregulation of PDGF-BB and Shh expression. Our preliminary results suggest that FOG-2, PDGF-BB and Shh may act together in a pathway required for coronary vasculogenesis. Future experiments will attempt to define more precisely both the mechanism by which FOG-2 regulates PDGF-BB expression and the other molecules involved in the FOG-2/PDGF-BB/Shh pathway.

5.25 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Exposure Prevents Epicardium Formation in Zebrafish

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Embryonic exposure to the environmental contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) disrupts cardiac development in a wide range of vertebrate species including fish, birds and mammals. The temporal window of TCDD sensitivity in zebrafish embryos coincides with the formation of the epicardium. We therefore hypothesized that TCDD-induced heart failure in zebrafish results from disruptions in epicardial development. Using *in situ* hybridization, histology and fluorescence immunocytochemistry in conjunction with confocal microscopy, we have found that early embryonic TCDD exposure prevents the formation of the proepicardium (PE) and epicardium. Exposure to TCDD after PE formation also disrupts epicardial development. An early response to TCDD exposure is reduced cardiac output; therefore we investigated whether impaired myocardial function alone was sufficient to disrupt epicardial development. To prevent myocardial contraction, we injected *silent heart* (*sih*) morpholinos (MO) and scored for PE and epicardium formation. The PE was present in *sih* MO injected larvae, however the epicardium did not form. Next, we impaired myocardial contraction pharmacologically by applying the reversible myosin ATPase inhibitor, 2,3-Butanedione 2-monoxime (BDM). Application of BDM during PE specification and growth (24 to 72 hours post fertilization; hpf) did not prevent the formation of the PE or the epicardium. However, application of BDM during epicardial cell migration (72 to 120

hpf and 96 to 120 hpf) stopped the further spreading of epicardial cells. Our results show that TCDD exposure prevents the formation of an essential layer of the vertebrate heart and that myocardial function is necessary for development of the epicardium in zebrafish.

5.27 Heterozygous deletion of Wilms' tumour-1 decreases fetal but not adult coronary artery development

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Congenital heart defects (CHD) are the most common human birth defect, occurring in approximately 5% of newborns. As a major cause of morbidity and mortality, a further understanding of the mechanisms underlying CHD is crucial. During heart development, Wilms' tumour-1 (*Wt1*) is expressed in epicardial cells and is critical for epicardium progenitor cell function, which gives rise to smooth muscle cells and fibroblasts that form coronary arteries. *Wt1*$^{-/-}$ mice have severe cardiac defects and are embryonic lethal. Conversely, *Wt1*$^{+/-}$ mice are viable and fertile but whether cardiac developmental defects occur due to a gene dosage effect remains elusive. We hypothesize that *Wt1*$^{+/-}$ mice will exhibit decreased coronary artery formation during development. Postnatal day one (P1) hearts were immunostained for α-smooth muscle actin and 3-D reconstructions were generated to evaluate coronary artery volume and morphology. *Wt1*$^{+/-}$ P1 mice exhibit significantly decreased coronary artery volume and coronary artery openings. There were no differences in heart size and total body weight. *Wt1*$^{+/-}$ mice had significantly lower expression of HIF1α at P1. By P3, both HIF1α and VEGFa were significantly increased. By P5, no significant differences were found in HIF1α and VEGFa. In adult *Wt1*$^{+/-}$ mice, no differences in coronary artery openings and capillary density were observed. We conclude that heterozygous deletion of *Wt1* decreases coronary artery development in fetal but not adult mice. Our data suggests that compensatory mechanisms develop postnatal in the *Wt1*$^{+/-}$ mice that lead to normal coronary artery formation in adulthood, potentially through the upregulation of HIF1α and VEGFa expression.

5.28 p38 MAPK and Src kinase function in TGFβ2 mediated epicardial cell invasion and differentiation.

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The epicardium undergoes proliferation, migration and differentiation into several cardiac cell types including smooth muscle that will contribute to the coronary vessels. Although the requirement for TGFβ family of ligands and receptors for epicardial epithelial to mesenchymal transition (EMT) have been recently characterized, the significance of downstream TGFβ signaling effectors have yet to be addressed. Both p38 MAP kinase and Src Kinase have recently been implicated in TGFβ signal transduction, but their potential roles in epicardial cell biology are unclear. Utilizing a primary murine epicardial cell model, we addressed the role of p38 and Src in TGFβ2 stimulated invasion and differentiation. Epicardial cells were treated with small molecule inhibitors and siRNA to p38 and Src, subsequently stimulated with TGFβ2, and allowed to differentiate. We demonstrate blocking p38 and Src activity results in inhibited TGFβ2 induction of pro-EMT genes such as CD44 and Hyaluronan Synthase 2, and block down-regulation of the TGFβ type III receptor. We show blocking p38 and Src leads to reduced TGFβ2 induction of epicardial Hyaluronan production. We evaluated the importance of p38 and Src kinase in TGFβ2 stimulated smooth muscle differentiation using a β-galactosidase reporter for smooth muscle actin 22α. Finally, blocking p38 and Src attenuates TGFβ2 induced invasion using a trans-well invasion assay and EMT in collagen gel cultures. Collectively, our data suggests p38 and Src kinase function in TGFβ2-mediated epicardial cell differentiation and invasion.

5.29 Somatopleural origin of a proepicardial cell population in the avian embryo

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The proepicardium (PE) develops at the venous pole of the tubular heart and in many vertebrates including the avian embryo PE development is asymmetric. Only the mesothelial cell cluster on the right side generates a tissue bridge via which proepicardial cells colonize the heart to form the epicardium. PE lateralization is under the control of a signaling pathway involving FGF8 and SNAI1 (Schlueter and Brand (2009) PNAS 106:7485-90). Expression analysis of TWIST1, which represents a potential target of the FGF8/SNAI1 pathway, revealed

transient expression in the right somatopleura and subsequently in the right sinus horn. Forced expression of SNAI1 on the left side caused ectopic TWIST1 expression suggesting that SNAI1 is upstream of TWIST1. The expression pattern of TWIST1 indicated that some PE cells might have a somatopleural origin. We performed cell fate experiments and observed a contribution of somatopleural cells to the sinus venosus and the PE. After forced expression of TWIST1 on the left side, somatopleural cells invaded the sinus venosus and the heart, suggesting that TWIST1 is able to enhance cell migration into the venous pole of the heart. Suppression of TWIST1 expression by shRNAi on the right side resulted in impaired proepicardial development and suppression of WT1 expression. Currently we are testing whether the loss of TWIST1 also leads to alterations in the cellular differentiation potential of proepicardial explants. These data suggest that the PE in the avian embryo is made up of distinct cell populations, which differ in their embryological origin.

5.30 Partial absence of pleuropericardial membranes in *Tbx18*- and *Wt1*-deficient mice

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The pleuropericardial membranes are fibro-serous walls that separate the pericardial and pleural cavities and anchor the heart inside the mediastinum. Partial or complete absence of pleuropericardial membranes is a rare human disease, the etiology of which is poorly understood. As an attempt to better understand these defects, we wished to analyze the cellular and molecular mechanisms directing the separation of pericardial and pleural cavities by pleuropericardial membranes in the mouse. We found by histological analyses that both in *Tbx18*- and *Wt1*-deficient mice the pleural and pericardial cavities communicate due to a partial absence of the pleuropericardial membranes in the hilus region, and we trace these defects to a persistence of the embryonic communication between these cavities, the pericardioperitoneal canals. Furthermore, we identify mesenchymal ridges in the sinus venosus region that tether the growing pleuropericardial membranes to the hilus of the lung, and thus, close the pericardioperitoneal canals. In *Tbx18*-deficient embryos these mesenchymal ridges are not established, whereas in *Wt1*-deficient embryos the final fusion process between these tissues and the body wall does not occur. We suggest that this fusion is an active rather than a passive process, and discuss the interrelation between closure of the pericardioperitoneal canals, lateral release of the pleuropericardial membranes from the lateral body wall, and sinus horn development.

Section 6: Early Heart Formation and Progenitors

S6.1 Quantitative analysis of polarity in 3D reveals local cell coordination in the embryonic mouse heart

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Several mutations in genes encoding members of the Planar Cell Polarity pathways impair heart morphogenesis. However, no asymmetric localization of such proteins has been reported in myocardial cells and it has remained unclear how the embryonic myocardium is polarized. We have now identified cell polarity markers in the mouse and aim to quantify the degree of coordination between myocardial cells in the embryonic heart. Classically, anisotropies which underlie organ morphogenesis have been quantified as a 2D problem, taking advantage of a reference axis. However, this is not applicable to the looped heart tube, which has a complex geometry. We have designed a 3D image processing framework, to map the regions in which cell polarities are significantly aligned. This novel procedure integrates multidisciplinary tools, including image segmentation, statistical analyses, axial clustering and correlation analysis. The result is a sensitive and unbiased assessment of the significant alignment of cell orientations in 3D, compared to a random axial distribution. We show that the axes of cell polarity, defined as the centrosome-nucleus axes, are frequently biased in a plane parallel to the outer surface of the heart, with a minor transmural component. This reflects the expansion of the cardiac chambers which precedes transmural thickening. Our study reveals that ventricular cells locally coordinate their axes over 100µm. This is in keeping with the growth of the myocardium, that we had shown by clonal analysis to be regionally oriented.

S6.2 Clonally dominant cardiomyocytes direct heart morphogenesis

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As vertebrate embryos develop into adulthood, their organs dramatically increase in size and change tissue architecture. Here, we used a multicolor clonal analysis to define the contributions of many individual cardiomyocytes as the zebrafish heart undergoes morphogenesis from a primitive embryonic structure into its complex adult form. We find that the single cardiomyocyte-thick wall of the juvenile ventricle forms by lateral expansion of several dozen cardiomyocytes into muscle patches of variable sizes and shapes. As juveniles mature into adults, this structure becomes fully enveloped by a new lineage of cortical muscle. Adult cortical muscle originates from a small number (~8) of cardiomyocytes that display clonal dominance reminiscent of stem cell populations. Cortical cardiomyocytes initially emerge from internal myofibers that in rare events breach the juvenile ventricular wall and expand over the surface. Our study illuminates dynamic proliferative behaviors that generate adult cardiac structure, revealing clonal dominance as a key mechanism that shapes a vertebrate organ.

S6.3 Gα_{13} is required for S1pr2-mediated myocardial migration by regulating endoderm morphogenesis

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During vertebrate development, bilateral populations of myocardial precursors migrate towards the midline to form the primitive heart tube, and this requires both their intrinsic properties and the appropriate environment, including endoderm. Disruption of myocardial migration leads to the formation of two bilaterally located hearts, a condition known as cardiac bifida. In zebrafish, signaling mediated by sphingosine-1-phosphate (S1P) and its cognate G protein-coupled receptor (S1pr2/Mil) controls myocardial migration. However, the underlying downstream mechanism remains poorly understood. Here we show that depletion of specifically the G protein isoform Gα_{13}, results in cardia bifida and tail blistering, defects reminiscent of those observed in embryos deficient for S1P signaling. Our genetic studies indicate that Gα_{13} acts downstream of S1pr2 to regulate myocardial migration through a RhoGEF/Rho-dependent pathway. Intriguingly, both cardiomyocytes and endoderm express S1pr2 and Gα_{13}, and cardiac-specific expression of Gα_{13} fails to rescue cardia bifida in the context of global Gα_{13} inhibition. Furthermore, we found that disruption of any component of the S1pr2/Gα_{13}/RhoGEF signaling pathway led to defects in endoderm morphogenesis that were correlated to cardia bifida, and that defects in both endoderm and myocardial migration resulting from Gα_{13} depletion were rescued coincidently. Overall, our findings represent the first evidence that S1pr2 controls myocardial migration through a Gα_{13}/RhoGEF-dependent pathway, and that it does so by regulating endoderm morphogenesis. Currently, we are investigating the mechanisms by which S1pr2/Gα_{13} signaling controls endoderm morphogenesis.

S6.4 A Cdc42 associated genetic network directs heart lumen formation and morphogenesis in Drosophila

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The *Drosophila* embryonic heart is a key model system for understanding heart specification. Our previous studies indicate that heart morphogenesis requires Slit/Robo signaling, a function conserved in vertebrates. The mechanisms by which these and other signals control heart formation are still unknown. Due to its role in membrane dynamics, we investigated the role of the small GTPase Cdc42 during *Drosophila* heart development and found it to be required for cardiac cell alignment and heart tube formation. Mutant or constitutively active Cdc42 in the developing heart causes improper cardioblast alignment and formation of multiple lumina, suggesting that Cdc42 is required during discrete steps of cardiogenesis. Cell polarity and filopodia dynamics are unaffected by loss of Cdc42, therefore Cdc42 might have a different role during heart morphogenesis. To understand the regulation of Cdc42 and to identify new genetic interactors, we performed a genetic screen for modifiers of Cdc42. We identified the tyrosine kinase Abelson (Abl), and the non-muscle myosin-II zipper to strongly interact with Cdc42. Abl itself shows a requirement for coordinated heart tube assembly, and Zipper exhibits a dynamic localization pattern during cardiogenesis, which depends on Cdc42 function, but is

independent of Slit/Robo. Activation of the formin-like protein Diaphanous (Dia) produced defects similar to activated Cdc42, indicating that control of cell shape changes is a key regulatory step during heart morphogenesis. Our data suggest a novel mechanism of cardiac morphogenesis involving Abl, Cdc42, Dia and Zip acting in a common pathway during cardiac cell shape changes and orchestrated heart lumen formation.

S6.5 Identification and characterization of a multipotent cardiac precursor

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Construction of the vertebrate heart is a complex process involving the regulated contribution of cardiac progenitor cells in a discrete spatial and temporal order. The precision required for this process is highlighted by the frequency of congenital heart defects. Understanding the identity and regulation of these progenitors is critical to understanding the origins of congenital heart defects and has the potential to lead to novel cell-based regenerative therapies for heart disease. Previous lineage tracing studies have predicted the existence of an early, multi-potent cardiovascular progenitor, but the identity of this progenitor has remained undefined. The transcription factor *Mesp1* has been postulated to mark early cardiac progenitors, however our lineage tracing results suggest that *Mesp1* labels a much broader domain of cardiac and non-cardiac mesoderm than initially reported. *Baf60c/Smarcd3*, a subunit of the BAF chromatin-remodeling complex, is expressed specifically in the heart and somites in the early mouse embryo. We have identified an early domain of *Smarcd3* expression, prior to formation of the cardiac crescent in a region of the late gastrula stage mouse embryo predicted to contain a cardiovascular progenitor population. Temporally-regulated lineage tracing of this population specifically labels both first and second heart field derived structures, including the endocardium, myocardium, and epicardium, and a population of cells within the anterior forelimb. Ongoing work will describe the precise temporal and spatial localization of *Smarcd3* relative to other markers of early cardiogenesis (*Tbx5*, *Isl1*, and *Nkx2-5*) as well as the clonal relationship among these early *Smarcd3*-expressing cells.

S6.6 Distinct origin and commitment of HCN4+ First Heart Field cells towards cardiomyogenic cell lineages

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Most of the mammalian heart is formed from two anatomically and molecularly distinct groups of cells of mesodermal origin termed the first and second heart fields (FHF and SHF). Whereas the SHF gives rise to the right ventricle, parts of the atria, and proximal region of the outflow-tract, the FHF gives rise to the left ventricle and remaining parts of the atria. Multipotent progenitors of the SHF are marked preferentially by Islet-1 expression, while a marker exclusive to FHF progenitors has not been identified. Here, we present Hcn4, a gene encoding the hyperpolarization-activated cyclic nucleotide-gated channel 4, as a new FHF marker expressed as early as pre-crescent stage. In-situ hybridization analysis revealed a dynamic expression of Hcn4 in the developing heart, with its strongest expression in the cardiac crescent and down-regulation by E9.5. HCN4-CreErt2 lineage tracing experiments confirm that the progeny of these cells give rise to FHF-derived structures of the heart. Surprisingly, these cells contribute primarily to cardiomyogenic cell lineages, suggesting that Hcn4+ FHF precursor cells are committed cardiomyogenic progenitors from their earliest detectable stage. Single cell clonal analysis using Hcn4+ FHF cells from both mouse embryos and differentiated mouse embryonic stem cells support this finding. Our data suggests a model of cardiogenesis, where the primary purpose of the FHF is to generate cardiac muscle to support the contractile activity of the primitive heart tube, whereas the contribution of SHF-derived progenitors to the heart is more diverse, leading to cardiomyocytes, smooth muscle and endothelial cells.

6.7 Dynamic Mesp1 regulation directs cardiac myocyte formation in embryonic stem cells

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Mesp1 sits on the top of the hierarchy of cardiac gene regulation network. It emerges at the onset of mesoderm formation and disappears at the cardiac crescent. The extracellular cue that triggers Mesp1, and how the Mesp1-expressing cells differentiate into cardiovascular cell lineages are largely unknown. In this study, we have crossed the $Mesp1^{Cre/+}$ and $Rosa^{EYFP/EYFP}$ mouse strains, and established an ES cell line with the genotype $Mesp1^{Cre/+}/Rosa^{EYFP/+}$. By isolating EYFP (+) cells on day 4 of an ES differentiation protocol, we characterized the early cardiac progenitors. These cardiac progenitors enriched cardiac mesoderm markers (Flk1, PDGFRa, dHand), cardiac transcription factors (Nkx2-5, Tbx5, Mef2c), and excluded pluripotent markers (Oct4, Sox2, Nanog) and nascent mesoderm markers (T, Fgf8). Among a group of growth factors, BMP2/4 greatly induced the prevalence of EYFP (+) cells, while Wnt3a and Activin had only marginal effects. EYFP(+) cells represented a sub-population of the Flk1(+)/PDGFRa(+) cells, which were previously demonstrated to differentiate into cardiac lineages. Surprisingly, short period of BMP4 treatment (day 0-2) led to the induction of EYFP(+) cells and subsequent cardiac myocyte formation, whereas extended BMP4 (day 0-4) led to even more EYFP (+) cells but without subsequent cardiac myocyte formation. By introducing a pCAGG-loxP-bgeo-polyA-loxP-Mesp1 cassette into this ES cell line, we achieved extended Mesp1 expression in EYFP(+) cells beyond the point that endogenous Mesp1 disappears, and proved that extended Mesp1 expression impaired cardiac myocyte formation. In summary, we have established an ES cell line that allows us to trace the fate of Mesp1-expressing cardiac progenitors. Using this line as the tool, we demonstrate that dynamic Mesp1 regulation directs cardiac myocyte formation in ES cells.

6.8 Human amniocytes contain subpopulations of stem cells that have a repressed cardiogenic status

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Amniocytes are an intriguing candidate source of stem cells for autologous repair of congenital heart defects (CHD) in humans. Despite mesodermal fate poising, amniocytes are resistant to differentiating into fully functional cardiomyocytes. Here, we provide evidence that cultured amniocytes are a heterogeneous subpopulation of stem cells expressing key pluripotency and self-renewal markers. Interestingly, qPCR analysis of 17 independent amniocyte isolates also detected the expression of the core cardiogenic factors Mef2c, Tbx5, and Nkx2-5, as well as the cardiac chromatin regulator Baf60c (Smarcd3). Gata4 is not expressed, but some individual isolates show signs of a low level of gene leakiness. However, Tbx5 and the early-to-mid cardiac markers Mesp1, Isl1, and Gata6 were consistently detectable by both qPCR and immunostaining. Stimulating amniocytes with signals known to promote differentiation of cardiac lineages failed to upregulate mRNA transcript or protein levels of core cardiogenic factors or generate cells that stain positive for cardiac troponin T (CTNT) and MF20 markers. To more precisely define the cardiogenic status of amniocytes, we performed next generation RNA-seq analysis on amniocytes. Genome-wide expression profiling reveals a paradoxical molecular signature of cardiac markers, indicating that the developmental status of amniocytes as true cardiac progenitors is incomplete, yet uniquely poised. Amniocytes strongly express multiple ES cell and mesodermal repressors that may prevent cells from progressing further down a cardiogenic lineage. These results indicate that despite an incomplete cardiogenic phenotype, direct transdifferentiation and gene knockdown approaches may allow us to overcome these barriers and drive them to a mature cardiac cell fate. Supported in part by Primary Children's Medical Foundation, T32HL007576 Cardiovascular Training Grant, and U01HL098179 (NHLBI Bench-to-Bassinet program)

6.9 The Homeobox Transcription Factor, Irx4, Identifies a Mutipotent, Ventricular-specific Cardiac Progenitor

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Cardiac progenitors have been presented as potential cell therapeutics, due to their cardiogenic potency. Iroquois homeobox protein 4 (Irx4) is the earliest known marker of ventricular myocardium differentiation, and the transcription factor is restricted to the ventricles throughout embryogenesis and into adulthood. Our goal is to identify a multipotent, ventricular-specific cardiac progenitor. We have targeted the 3' end of the Irx4 locus using a recombineering approach to insert fluorescence (mCherry), bioluminescence (luciferase), and antibiotic resistance (hph) cassettes. Six mESC clones were properly targeted $Irx4^{luc\text{-}mCh\text{-}hph/wt}$ ES cells. RT-PCR, western blot analysis, and immunofluorescence assays demonstrated the functional integration of the reporters during embryoid body (EB) differentiation. Following 4 days of differentiation of the $Irx4^{luc\text{-}mCh\text{-}hph/wt}$ cells in EBs, selection with hygromycin was carried out for 2 days, and day 6 cells were plated onto STO cell feeder layers for expansion. Selected $Irx4^{+}$ cells are proliferative, expressing the cell cycle antigen, Ki67, and could be passaged more than 12 times. The $Irx4^{+}$ cells express cKit, Flk1, and CXCR4 on the cell surface. Selected $Irx4^{+}$ cells demonstrate cardiovascular potency when re-aggregated and differentiated in hanging drops resulted in ventricular myocyte-enriched cell preparations (65±3.7% $cTnT^{+}$; 67±2.6% $Myl2^{+}$, 22±3.1% $SmMHC^{+}$, and 6±2% $CD31^{+}$ cells. The selected $Irx4^{+}$ population represents the first ventricular-specific multipotent progenitor population identified and holds promise for generating all cardiovascular lineages necessary to reconstitute infarcted myocardium.

6.10 Modeling the vascular phenotype of Williams-Beuren syndrome using induced pluripotent stem cells

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Elastin is essential for arterial morphogenesis. Elastin haploinsufficiency in Williams-Beuren syndrome (WBS) leads to increased vascular smooth muscle cell (SMC) proliferation and vascular stenoses. We investigated the role of elastin in proliferation and differentiation of SMCs derived from human embryonic stem cells (hESCs), and WBS patient-derived induced pluripotent stem cells (hiPSCs). Human iPSCs were reprogrammed from skin fibroblasts from a WBS patient and BJ healthy control using 4-factor retrovirus reprogramming. hESCs and hiPSCs underwent directed differentiation to generate smooth muscle cells (SMCs). Differentiated cells were treated with a peptide or an anti-proliferative drug for 6 days. mRNA and protein expression of elastin, smooth muscle α-actin (SMA), Ki67 (proliferation marker) was assessed by high-content imaging, qRT-PCR, and flow cytometry. Vascular tube formation was assessed by 3D matrigel assays. Treatment of hESC-derived SMCs increased elastin expression, increased SMA+ cells, and reduced SMC proliferation. The reprogrammed fibroblasts expressed pluripotency genes and differentiated into all germ layers. SMC differentiation was confirmed by SMA, SM22a, myocardin, smoothelin expression and by contractile response to carbachol. When compared to BJ SMCs, WBS SMCs showed lower expression of elastin protein and mRNA which increased with treatment. WBS cells showed impaired SMC differentiation, increased SMC proliferation, and impaired vascular tube formation compared to BJ cells. Treatment with an elastin ligand and a candidate drug reduced SMC proliferation, enhanced SMC differentiation, and enhanced tube formation. In conclusion, WBS iPSCs demonstrated increased SMC proliferation and impaired SMC differentiation which was partially ameliorated with treatment. Our study provides an *in vitro* platform for modeling vascular disorders and future drug screens.

6.11 Galnt11 is a Novel Glycosylation Factor that Interacts with Notch to Affect Left-Right Development and Heart Formation

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Heterotaxy is a disease of abnormal left-right (LR) body patterning associated with congenital heart disease that has a significantly increased prevalence in patients with ciliopathies. Through copy number variant analysis of Heterotaxy patients, *Galnt11* was identified as a glycosylation factor important in human LR development. Morpholino (MO) knockdown and mRNA overexpression of *Galnt11* in *X. tropicalis* results in abnormal heart

looping, and the splice-site MO phenotype can be rescued with GALNT11 mRNA. Sided MO injections at the 2-cell stage revealed that *Galnt11* is only required on the left side for proper heart looping. Knockdown and overexpression also results in abnormal *Coco* and *PitX2* (early LR markers) expression patterns via in situ hybridization. *Galnt11* is expressed in the *X. tropicalis* gastrocoel roof plate (GRP) and kidneys, and in mouse Galnt11 protein localizes to the crown cells surrounding the pit cells of the node. This points to a ciliary role of *Galnt11*, which we investigated on the *X. tropicalis* ciliated epidermis. *Galnt11* knockdown yields an increase, while Galnt11 overexpression yields a decrease in cilia clump density, which is a known Notch phenotype. To further investigate the relationship between Galnt11 and Notch, we were able to rescue abnormal *PitX2* expression in *Galnt11* morphants by injecting mRNA of two Notch components downstream of Notch receptor, *Notch-ICD* and *Su(H)-Ank*, but not by injecting *Delta*, a Notch ligand. These results indicate that Galnt11 is a glycosylation factor that interacts with Notch, possibly at the level of Notch receptor, to affect proper LR development and heart formation.

6.12 Zic3 is required in the migrating primitive streak for node morphogenesis and left -right patterning

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In humans, loss of function mutations in *ZIC3* cause X-linked heterotaxy, a disorder characterized by abnormal left-right asymmetry of organs and frequent cardiovascular malformations. *Zic3* is expressed ubiquitously during critical stages for left-right patterning but its later expression in the developing heart remains controversial and the molecular mechanism(s) by which it causes heterotaxy are unknown. *Zic3* null mice recapitulate the human heterotaxy phenotype but also have early gastrulation defects, complicating an assessment of the role of *Zic3* in cardiac development. To define the temporal and spatial requirement for *Zic3* in left-right patterning, we generated conditional *Zic3* mice and *Zic3*-LacZ-BAC reporter mice. The latter provide compelling data indicating that *Zic3* is expressed in the mouse node. We hypothesized that *Zic3* expression in node cells is required for proper left-right asymmetry. To address this question, *Zic3* was deleted in each cell type of the node using a conditional loss of function approach. Surprisingly, *Zic3* deletion in the node results in viable, phenotypically normal mice. However, immunohistochemistry and scanning electron microscopy indicate abnormal node morphology in *Zic3* null mice. We observed similar node dysplasia when *Zic3* was deleted from the migrating mesoderm and primitive streak at E7.5. At later stages, heart looping defects were observed. Interestingly, mice were viable when *Zic3* was deleted from the heart compartment and heart progenitors. These results contrast with the accepted paradigm that left-right asymmetry is initiated at the node in mice and implicate *Zic3* in earlier signaling events affecting node formation and subsequent node function. This work was funded by RO1 HL088639 from the National Institutes of Health (S.M.W.) and NIH T32 HL007752-16 Pulmonary and Cardiovascular Developmental Training Grant (J.A.W.)

6.13 The Vacuolar ATPase Accessory Protein Atp6ap1 Controls Heart Laterality in Zebrafish

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Establishing left-Right (LR) asymmetry, or laterality, of the primitive heart tube is critical for subsequent cardiac form and function. Small molecule screens have identified a role for the Vacuolar Type ATPase (V-ATPase) in establishing heart laterality, but the underlying mechanisms remain unclear. The V-ATPase is a multi-subunit proton pump in membranes that maintain organelle and cellular pH by pumping protons into the organelle lumen and out of the cellular cytoplasm. In the zebrafish embryo, the V-ATPase accessory protein Atp6ap1 is prominently expressed in a transient organ called Kupffer's vesicle (KV). Motile cilia that project into the KV lumen generate an asymmetric fluid flow that is required for normal cardiac laterality. Depletion of Atp6ap1 using antisense morpholinos caused heart LR defects and disrupted the formation of KV. Atp6ap1 knockdown significantly decreased the length and number of KV cilia and reduced KV lumen size. These KV defects were rescu ed by ectopic Atp6ap1 expression. Interestingly, over-expression of Atp6ap1 also disrupted KV development and heart laterality, suggesting tight control of V-ATPase activity is essential. Consistent with this idea, interfering with V-ATPase function via morpholino knockdowns of V-ATPase subunits or treatments with the small molecule V-ATPase inhibitor concanamycin resulted in similar KV and cardiac LR defects. These results indicate V-ATPase function regulates KV formation, a critical step in establishing laterality of the heart. Next, we will elucidate mechanisms by which the V-ATPase regulates KV development and heart LR asymmetry

by examining potential connections between V-ATPase activity and the Notch, FGF and Wnt signaling pathways.

6.14 A potential role for IGFBP5 in left-right cardiac specification

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Heterotaxy (Htx) is a condition where the normal left-right (LR) asymmetry is not properly established. Cilia present at the LR organiser establish the LR axis. Using a genome-wide analysis of copy number variations (CNVs) in Htx patients we have identified a genomic deletion in the Insulin Growth Factor Binding Protein (IGFBP5) a novel gene previously unrecognised in LR patterning. We have used Xenopus as a model to characterise the role of IGFBP5 in cardiac development. Overexpression of IGFBP5 mRNA on the left side of the two-cell stage embryo resulted in 22% abnormal A or L looping ($p<0.001$) whereas right side injected embryos had no significant difference in abnormal cardiac looping compared to GFP injected controls. Furthermore IGFBP5 overexpression led to loss of left side Pitx2 expression (early marker of LR development) in 42% of left side injected two-cell stage embryos compared to unilateral left sided expression in GFP injected controls. Whole-mount immunofluoresence staining for acetylated tubulin demonstrated a reduced number of epidermal cilia clumps in 40% of left sided injected two-cell stage embryos compared to GFP injected controls. Preliminary data using morpholino knockdown studies of IGFP5 knockdown in one-cell stage embryos showed 13.8% abnormalities in cardiac looping compared to 0% in GFP injected controls ($p<0.05$). We conclude that IGFBP5 is involved in establishing a LR body axis in an asymmetrical dependent manner potentially signalling through ciliated cells.

6.15 Cell behaviors driving early heart elongation and looping

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Establishing left-right (LR) asymmetry in the developing heart is essential for correct heart positioning and function in vertebrates. Although the molecular mechanisms that establish LR asymmetry are known, how molecular information is interpreted in individual cells to drive morphogenesis remains unknown. To address this, we have analyzed the initial LR asymmetric morphogenesis of the early heart rudiment, a process called C-looping. In C-looping, the primitive heart--a short, symmetric straight tub--undergoes rapid elongation and becomes C-shaped. To determine the mechanism of oriented looping, we performed morphological and histological analyses. We found that C-looping is accomplished by asymmetric tissue growth between the left and right heart rudiments. To address what cellular events drive asymmetric growth, we next performed cell tracing using fluorescent dyes and time-lapse microscopy. Surprisingly, we found that labeled cell clusters dramatically elongate in tandem with heart tube elongation and increased their length 5 times in 18 hours. The speed of cluster elongation is sufficiently fast as to seemingly not be due to solely proliferation, suggesting that the heart tube elongates by cell rearrangement such as intercalation. We further compared the manner of deformation of labeled cell clusters in the left and right rudiments and will discuss differences in cell properties between left and right heart tissues that control differential growth and orient the direction of loop formation.

6.16 RARαb1 deficient embryos have increased RA signaling that promotes cardiomyocyte specification in zebrafish

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One function of retinoic acid (RA) signaling during development is to restrict cardiomyocyte (CM) specification. Understanding the transcriptional mechanisms RA signaling uses to restrict CM specification will aid our ability to precisely direct CM differentiation in vivo and in vitro. In vertebrates, however, the specific RA receptors (RARs) required to restrict CM specification have not been reported. Here, we find embryos deficient for RARαb1, a previously unrecognized conserved zebrafish RARα splice variant, have enlarged hearts with increased CM number. Surprisingly, we find that expression of hoxb5b, which RA signaling positively regulates and was found to restrict atrial cell number, was increased in RARαb1 deficient embryos. Importantly, depletion of hoxb5b is able to rescue the enlarged hearts found in RARαb1 deficient embryos, suggesting that the

increase of hoxb5b at least in part causes the increase in the CM number. In contrast to inhibiti ng RA signaling components using other methods, examination of additional RA responsive genes and RARs indicates that their expression is also increased in RARαb1 deficient embryos, suggesting that the loss of RARαb1 results in a general increase of RA signaling. Although effects were not observed on the development of the heart size or CM number, similar effects on RA signaling components were found in RARαb2 deficient embryos. Altogether, our results suggest an intriguing model where depletion of RARαb1 results in a feedback loop that induces modest increases in RA signaling, which ultimately promote increased CM specification.

6.17 Coordinated myocardial movements during progressive stages of heart tube assembly and early looping.

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Transformation of planar cardiac progenitor fields into a progressively more complex structure, the heart, involves a series of cellular and tissue movements in three dimensions. However, the driving forces behind these movements are not fully understood. We investigated the motion of fluorescently tagged myocardial progenitors in live quail embryos using time-lapse imaging. The fibronectin ECM environment for myocardial movement was visualized in vivo with microinjected fluorescent antibodies, while endocardial progenitors were labeled with transgenic expression of Tie1::H2B-YFP. The imaging period encompassed the motion of myocardial progenitors from primary heart field(s) to the midline and continued through early heart looping stages. We determined the relative importance of directed cell autonomous (relative to the ECM) motility versus convective tissue movements in the assembly of a myocardial layer of the cardiac tube using object tracing and particle image velocimetry. These quantitative data indicate the contribution of cell autonomous motility displayed by myocardial progenitors is limited, with convective tissue movements playing a major role in their arrival at the midline. Further, concomitantly with the onset of cardiac tube bending, caudal myocardial primordia underwent a coordinated displacement in the posterior-ventral direction (“rotation”), suggesting a possible connection between these processes. Interestingly, this rotation was abrogated following the introduction of exogenous VEGF165, but proceeded in separated primordia following incision-induced cardia bifida. Ongoing studies are underway to assess the effects of myocardial-specific expression of dominant negative and constitutively active isoforms of RhoA on rotation and to further elucidate the cellular and tissue mechanisms driving this bulk movement.

6.18 Primary cilia control proliferation of myocardium during early cardiogenesis

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Cardiac contraction influences morphogenesis, however, the sensing mechanism remains unclear. Cardiac cilia are found in the embryonic mouse heart, and embryos lacking cilia have thinned compact myocardium. We evaluated myocardial proliferation in mouse embryos with mutations affecting ciliogenesis: Kif3A-/- and IFT20-/-. Hearts were isolated from 11-20somite stage embryos, mitotic cells labeled with anti-Phospho histone, imaged using optical sectioning and total and PH3+ cells were counted. At 11 somites, cell counts in WT, Kif3A-/- and IFT20-/- are the same. By 20 somite stage Kif3A-/- and IFT20-/- hearts have 30% fewer cells than WT. PH3 staining shows that the percent PH3 positive myocardial cells is unchanged between WT, Kif3A-/- and IFT20-/-. However, the ratio of myocardial cells in prophase, metaphase and anaphase differed significantly: Kif3A-/- and IFT20-/- embryo hearts had significantly fewer myocardial cells that had progressed from prophase to metaphase and anaphase than WT. These data indicate that failure of myocardial cells to progress through the cell cycle contributes to myocardial thinning in embryos with defective cilia. The proliferative defects observed in Kif3A-/- and IFT20-/- hearts coincided with onset of contraction. To evaluate the effect of contraction on myocardial proliferation, we evaluated hearts of Ncx-/- embryos, which do not contract. Cell counts and PH3 staining in the Ncx-/- hearts were indistinguishable from cilia-defective hearts. These data suggest that contraction provides a signal for myocardial proliferation, and support the hypothesis that cilia in the myocardium may function as sensors for contraction, and that they have a role in control of myocardial proliferation.

6.19 Functional significance of SRJ domain mutations in CITED2

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CITED2 is a transcriptional co-activator with 3 conserved domains shared with other CITED family members and a unique Serine-Glycine Rich Junction (SRJ) that is highly conserved in placental mammals. Loss of Cited2 in mice results in cardiac and aortic arch malformations, adrenal agenesis, neural tube and placental defects, and partially penetrant defects in left-right patterning. Non-synonymous mutations in CITED2 have previously been detected in small studies of human congenital heart disease (CHD). By screening 1126 sporadic CHD cases and 1227 controls, we identified 19 variants, including 5 unique non-synonymous sequence variations (T166N, R92G, N62S and A187T, G180-A187del) in patients. Many of the CHD-specific variants identified in this and previous studies cluster in the SRJ domain. Transient transfection experiments indicated that T166N mutation impaired TFAP2 co-activation function. In order to investigate the functional significance in vivo, we generated a T166N mu tation of mouse Cited2. We also used PhiC31 integrase-mediated cassette exchange to generate a Cited2 knock-in allele replacing the mouse Cited2 coding sequence with human CITED2 and with a mutant form deleting the entire SRJ domain. Mouse embryos expressing only CITED2-T166N or CITED2-SRJ-deleted alleles surprisingly show no morphological abnormalities, and mice are viable and fertile. These results indicate that the SRJ domain is dispensable for these functions of CITED2 in mice and that point mutations and deletions clustering in the SRJ region are unlikely to be the sole cause of the malformations observed in patients with sporadic CHD. We suggest that additional factors are required to cause CHD in such patients.

6.20 Distinct phases of Wnt/ß-catenin signaling direct cardiomyocyte formation in zebrafish.

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Normal heart formation requires distinct phases of canonical Wnt/β-catenin (Wnt) signaling. Understanding the mechanisms by which Wnt signaling drives correct cardiac formation *in vivo* is critical for understanding the formation of the heart as well as essential to studies developing stem cells into cardiomyocytes (CM) *in vitro*. Here, we investigated the roles of Wnt signaling using heat-shock inducible transgenes which allow us to increase or decrease Wnt signaling in the embryo. During the first 24 hours of development, we find three distinct phases during which Wnt signaling modulates CM formation. Wnt signaling has previously been implicated in mesoderm specification as well as regulating the pre-cardiac mesoderm. Here, we have also identified a later role after CM differentiation has initiated, in which Wnt signaling is necessary and sufficient to promote the differentiation of atrial cells. This study also extends the previous studies on Wnt signaling during mesoderm specification and in the pre-cardiac mesoderm in zebrafish. Interestingly, we define a new role for Wnt signaling in the pre-cardiac mesoderm where Wnt is sufficient to prevent cardiac cell differentiation, which leads to Caspase-3 independent cell death. Together with a previously described later role of Wnt signaling in restricting ventricular CM proliferation, our results indicate that there are four distinct phases of Wnt signaling during the first 3 days of zebrafish development that allow for the proper formation of the heart.

6.21 Defining Paraxial mesoderm contribution to the heart in chick embryo

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The myocardium is derived from the splanchnic mesoderm sheet (SMS) directly where the endocardium is derived from cells that delaminate first from the SMS towered the endoderm sheet and then fuse medially forming a tube. The SMS moves as a sheet and fuse medially around stage HH9-10, it continues till the entire myocardium is formed around stage HH18-19, around stage HH12 the middle of the sheet of the right and left side fuse together dorsally giving rise for the first time to the formation of the heart tube, from now on the contribution to the two heart poles (outflow and inflow) becomes independent. The SMS continues laterally with paraxial mesoderm and so the question is how much if any of the paraxial mesoderm does contribute to the myocardium. Using crystal dye that allow lebeling for few cells diameter at one time preventing unwanted

diffusion but can label hundreds of cells that pass by over longer time, cells of the paraxial mesoderm at different distances from SMS a nd at different stages (HH8-HH13) where labeled and followed till later stages (HH18-19) and their contribution where visualized either by cryo-sectioning or special clearing of the embryo. The results unequivocally demonstrate that in addition of the splanchnic mesoderm, a small adjacent group of cells (5-6 cells diameter) behave like a source and contribute quite consistently to the forming myocardium with the complete embryonic ventral closure.

6.22 Mouse-chick chimeric transplantation reveals the regional contribution of the cell population arising from the inflow tract to cardiac chamber formation

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Previously, we identified a distinct cell population defined by the expression of Endothelin type-A receptor (Ednra) in the mouse early inflow region. This population mainly contributes to the left ventricle and atria during E8.25 to E9.5. To facilitate lineage analysis of this cell population during cardiac development, we established a mouse-chick chimera model. Cardiac inflow tissues corresponding to *Ednra*-expressing region were excised from E8.25 (5- to 7-somite stage) mouse embryos carrying the *Mesp1-Cre*;R26R reporter gene and transplanted into the same regions of 2-day-old chick embryos without removing their own tissues. After 3 days, b-galactosidase-positive cells derived from the mouse graft were detected in the chick heart, especially in the left ventricle, atria, venous valves and ventricular septum. In the left ventricle, b-galactosidase /myosin heavy chain (MHC) double-positive cells were frequently observed. The distribution patterns of b-galactosidase-positive cells appeared to partially reflect similar to those of *Ednra*-expressing cells. In addition, in vitro culture showed *Ednra*-EGFP-labeled colonies expressing MHC arising from the inflow region. These results indicate the regional contribution of the inflow cell population characterized by *Ednra* expression to chamber formation. This mouse-chick chimera system is useful for lineage tracing and fate mapping of genetically-labeled mouse cardiogenic tissues.

6.23 Identification of a Novel Developmental Mechanism in the Generation of Mesothelia.

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Mesothelial cells form the surface layer of all coelomic structures and are essential to organ function. During development, these cells undergo an epithelial to mesenchymal transition (EMT) to provide the precursors for the vasculature and stromal cells to all coelomic organs investigated to date. Furthermore, in the adult, mesothelial cells stimulated by disease or injury retain the ability to undergo EMT to generate fibroblasts and vascular smooth muscle cells mimicking their developmental behavior. Despite the broad contribution of this cell type to developing organs and adult disease, our current understanding of the genesis of this cell type is confined to a single organ, the heart, in which an exogenous population of cells, the proepicardium, migrates to and over the myocardium to give rise to the cardiac mesothelium and coronary vasculature. It is unknown whether this pattern of development is specific to the heart or applies broadly to other coelomic organs. Using two independent long term lineage tracing studies, we demonstrate that mesothelial progenitors of the intestine are intrinsic to the gut tube anlage. Furthermore, a novel chick-quail chimera model of gut morphogenesis reveals these mesothelial progenitors are broadly distributed throughout the gut primordium and are not derived from a localized and exogenous proepicardium-like source of cells. These data demonstrate an intrinsic origin of mesothelial cells to a coelomic organ and provide a novel mechanism for the generation of mesothelial cells.

6.24 Characterization of the pro-cardiac activity conferred by Gata5 and Smarcd3b in the zebrafish embryo

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In the zebrafish cardiac progenitors are first found in the blastula in several rows of cells at the embryonic margin. During gastrulation these cells ingress and move to the anterior lateral plate mesoderm (ALPM) where they will then migrate towards the midline and fuse to form a heart tube. How cardiac progenitors specifically migrate to the ALPM, how they become specified to the cardiac lineage and how they are maintained are all

questions that remain unanswered. The transcription factor Gata5 and chromatin re-modeling complex sub-unit Smarcd3b are able to direct non-cardiac cells to migrate to the ALPM and form part of the heart in the developing zebrafish embryo. Studying how this pro-cardiac activity is accomplished may help address some of these outstanding questions. A conditional version of Gata5 been utilised to determine the temporal requirement of it in this phenomena. Gata5 activity is found to be necessary early in gastrulation for pro-cardiac activity which may suggest a novel role for it in heart development. In order to fully characterize the molecular mechanisms which underlie this activity transcriptional profiling has been performed on *gata5/smarcd3b* over-expressing cells during gastrulation. This has revealed a unique set of transcripts in these cells of which further analysis may reveal novel regulators of cardiac progenitor development.

6.25 Interrogating Cardiovascular Morphogenesis in Zebrafish Through Small Molecule Perturbation

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Cardiogenesis involves the coordination of complex cell behaviors necessary to generate the three-dimensional structure of the heart. For example, heart tube assembly requires the migration of bilateral populations of cardiomyocytes to the midline where they meet and merge to form a cardiac ring through a process called cardiac fusion. Several genes are known to influence cardiac fusion, yet our understanding of the regulation of this aspect of cardiac morphogenesis remains incomplete. We aim to identify additional mediators of cardiac fusion by screening for small molecules that can alter the dimensions of the cardiac ring. So far, our pilot screen has identified four compounds of interest, and we have focused on two, ketanserin and scopoletin, that expand the shape of the cardiac ring. Treatment with ketanserin, a serotonin receptor antagonist, and treatment with scopoletin, a coumarin derivative, both seem to disrupt cell movements during cardiac fusion, resulting in defects in myocardial and endocardial tube assembly. In addition, ketanserin treatment disrupts atrioventricular canal differentiation, whereas scopoletin treatment leads to an increased number of erythrocytes. Therefore, we propose that ketanserin and scopoletin disrupt two distinct pathways with similar influences on cardiac fusion. We are currently investigating the molecular mechanisms by which ketanserin and scopoletin affect cardiovascular development.

6.26 Snx30: a new negative regulator of Wnt/β-catenin signaling identified in the frog.

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Wnt/β-catenin signaling is a key regulator of cardiogenesis that occurs in distinct phases of activation and repression. Although secreted antagonists of Wnt/β-catenin signaling have been identified, intracellular regulation of this pathway is poorly understood. In this study, we identified *Snx30*, a new repressor of Wnt/β-catenin signaling required during early cardiac specification in Xenopus laevis. *Snx30* is a member of the sorting nexin family that regulate intracellular trafficking of membrane-bound proteins. In Xenopus embryos, Snx30 was expressed in three phases, the strongest peak occuring before gastrulation and limited to the animal pole of the blastula. This triphasic pattern of expression was similar to that observed in differentiating human cardiomyocytes. Also, expression in human fetal heart was at least two-fold stronger than in adult heart. Knockdown of *Snx30* in Xenopus embryos caused a broad phenotype, with 60% mortality, developmental delay appearing at the onset of gastrulation and generalized anterior defects. A closer look revealed upregulation of classical Wnt/β-catenin target genes, *Xnr3* and *Siamois*, as well as cardiac defects. Data from immunohistochemistry and subcellular fractionation in HEK293 cells suggest that SNX30 localizes to early endosomes. In conclusion, we propose that *Snx30* functions as a repressor of Wnt/β-catenin signaling during the inhibitory phases required for early cardiac specification. This role may be accomplished by trafficking of Wnt receptors towards an intracellular degradation route, as seen in other sorting nexins, but this requires further study. Understanding the early steps of cardiogenesis could help develop better techniques to produce functional cardiomyocytes used in stem cell therapy.

6.27 Vascular Endothelial And Endocardial Progenitors Differentiate As Cardiomyocytes In The Absence Of Etsrp/Etv2 Function

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Previous studies have suggested that embryonic vascular endothelial, endocardial and myocardial lineages originate from multipotential cardiovascular progenitors. However, their existence in vivo has been debated and molecular mechanisms that regulate specification of different cardiovascular lineages are poorly understood. An ETS domain transcription factor Etv2/Etsrp/ER71 has been recently established as a crucial regulator of vascular endothelial differentiation in zebrafish and mouse embryos. In this study, we show that etsrp-expressing vascular endothelial/endocardial progenitors differentiate as cardiomyocytes in the absence of Etsrp function during zebrafish embryonic development. Expression of multiple endocardial specific markers is absent or greatly reduced in Etsrp knockdown or mutant embryos. We show that Etsrp regulates endocardial differentiation by directly inducing endocardial nfatc1 expression. In addition, Etsrp function is required to inhibit myocardial differentiation. In the absence of Etsrp function, etsrp-expressing endothelial and endocardial progenitors initiate myocardial marker hand2 and cmlc2 expression. Furthermore, Foxc1a function and interaction between Foxc1a and Etsrp is required to initiate endocardial development, but is dispensable for the inhibition of myocardial differentiation. These results argue that Etsrp initiates endothelial and endocardial, and inhibits myocardial, differentiation by two distinct mechanisms. Our findings are important for the understanding of genetic pathways that control cardiovascular differentiation during normal vertebrate development and will also greatly contribute to the stem cell research aimed at regenerating heart tissues.

6.28 Nkx2-5 mediates differential cardiac differentiation through interaction with Hoxa10

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Nkx2-5 has long been utilized as a marker of early cardiac progenitor cells. Recent studies have documented that Nkx2-5 positive cells are not limited to the cardiac lineage but can give rise to endothelial and smooth muscle lineages. Other work has elucidated that, in addition to promoting cardiac development, Nkx2-5 plays a larger role in mesodermal patterning although the transcriptional networks that govern this developmental patterning are unclear. By profiling early Nkx2-5 positive progenitor cells, we discovered the progenitor pools of the bisected cardiac crescent are differentiating asynchronously and the relative acceleration of the left side is Nkx2-5 dependent. Surprisingly, posterior Hox genes, Hoxa9 and Hoxa10, were expressed in the right side, independently of Nkx2-5. Hoxa10 has been shown to play a role in regulating hematopoietic proliferation and differentiation but posterior Hox genes have never been implicated in early mammalian cardiac development. In differentiating ES cells, Hoxa10 is expressed in the earliest Nkx2-5 positive progenitors and ectopic Hoxa10 expression reduces structural cardiac gene expression. This data suggests a model in which Nkx2-5 mediates differential timing of cardiac differentiation through an unprecedented interaction with a posterior Hox gene.

6.29 Selective loss of Vcan Proteins Causes Heart Defects and Alters Collagen Deposition.

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The requirement for the proteoglycan versican (Vcan) to early heart formation was clearly demonstrated by the Vcan null mouse called heart defect (hdf). Total absence of the Vcan gene is lethal at a stage prior to the heart's pulmonary/aortic outlet segment growth. This creates a challenge for determining Vcan's significance in the forming valve precursors and vascular wall of the pulmonary and aortic trunks. Fortunately, 3 mouse models of partial Vcan depletion do survive to birth. Both the hdf hemizygous and halpn1 null mice have reduced (50%) levels of Vcan expression and similar heart defects. The third model, a deletion of exon 7 in the Vcan gene also

has reduced levels (50%) of Vcan expression due to the complete lack of two of the 4 alternative Vcan forms. Comparison of the 3 models showed similarities in heart defects such as, ventricular septal defects and a thin myocardium. However, the exon 7 null also has an additional spectrum of cardiac outlet defects that include changes in valve precursors and walls of the pulmonary and aortic trunks. We compare the changes in E13.5 and neonate hearts by biochemical and histological analysis including Hsp47 western blot, sircol quantification, and pircrosirius red staining patterns. A change in abundance and banding pattern of the collagen fibril chaperone protein, Hsp47 was measured. Hearts lacking Vcan exon 7 have a 2.5 fold reduction of insoluble compared to soluble collagen ($p<0.05$) and an altered histological pattern of collagen deposition in the forming valve leaflets and walls of the aorta and pulmonary arteries. Our study suggests that a splice variant of exon 7 in Vcan plays a role in regulating collagen deposition during aortic and pulmonary valve remodeling and arterial wall formation that underlie the outlet defects found in the Vcan exon 7 null hearts. **Acknowledgements:** NIH NHLBI HL66231 (CHM); Janey Briscoe Center for Excellence in Cardiovascular Research (CJLS).

6.30 Smad- and miR-regulated VEGF suppression in the OFT development

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Epithelial mesenchymal transformation (EMT) is a very important event happened during cardiogenesis. BMPs have been implicated to play a pivotal role in this process, but little is known about the mechanism. To address the role of BMP signaling, we deleted *Bmp4* and *Bmp7* in the outflow tract (OFT) using the *Mef2c-Cre* driver. We found that the OFT of *Bmp4/7* double conditional knockout (dCKO) embryos exhibits EMT deficiency which is associated with up-regulation of *VEGF*. Luciferease analysis and *in vivo* ChIP indicated that expression of *VEGF* is repressed by intercellular components of BMP pathway, Smad, strongly suggesting *VEGF* is a direct target of BMP signaling. In addition, *VEGF* is tightly controlled by *miR-17-92* via 3' UTR silence showing that regulation of *VEGF* is mediated by *miR-17-92*-dependent BMP signal pathway. EMT defect in *BMP4/7* dCKO was partially rescued by VEGF antagonist, sFlt in explants culture and by overexpression of *miR-17-92 in vivo*. This study unveils that Smad- and miR-depenent regulation of *VEGF* is crucial for the OFT development.

Section 7: Chamber Development

S7.1 *irx1a* Acts Downstream of *nkx* Genes in Maintaining Cardiac Chamber Identity in Zebrafish

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Maintenance of the features characteristic of each cardiac chamber is essential to ensure efficient and organized contractility of the heart. Despite the importance of preserving chamber-specific characteristics, the regulatory mechanisms guiding this process are yet to be uncovered. Here, we show that Nkx transcription factors are necessary to sustain ventricular attributes through repression of atrial identity. We recently isolated mutant alleles for *nkx2.5* and *nkx2.7,* two zebrafish homologs of *Nkx2-5*. *nkx2.5*$^{-/-}$ mutants exhibit a decrease in ventricular and an increase in atrial size. Loss of *nkx2.7* enhances this phenotype: *nkx2.5*$^{-/-}$*;nkx2.7*$^{-/-}$ mutants display a complete absence of the ventricular chamber. The initial numbers of ventricular and atrial cardiomyocytes in mutant embryos appear normal. Yet, ventricular cardiomyocytes are lost and atrial cells are gained during chamber emergence. Additionally, analysis of chamber-specific gene expression patterns highlights a switch from a ventricular marker, *vmhc*, to a marker of atrial identity, *amhc*, suggesting transdifferentiation. Furthermore, our studies indicate that the Iroquois transcription factor Irx1a acts downstream of *nkx* genes. Ventricle-specific expression of *irx1a* is downregulated in *nkx2.5*$^{-/-}$ and *nkx2.7*$^{-/-}$ mutants and is absent in *nkx2.5*$^{-/-}$*;nkx2.7*$^{-/-}$ mutants. Inhibition of *irx1a* function leads to atrial expansion and ventricular diminution, uncovering its important role in regulating cardiac chamber proportionality. Together, our results demonstrate a pivotal role for *nkx* genes in maintenance of cardiac chamber identity and underscore a previously unappreciated function of *irx1a*. These findings have the potential to uncover etiologies of congenital heart disease in patients with *NKX2-5* mutations and to direct innovations in cardiac regenerative medicine.

S7.2 Zebrafish second heart field development relies on early specification of progenitors and nkx2.5 function.

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We recently reported that latent TGFβ binding protein 3 (ltbp3) marks the zebrafish anterior second heart field (SHF) that contributes myocardium to the distal ventricle and three cardiovascular lineages to the outflow tract (OFT). However, SHF expression of ltbp3 initiates at the arterial pole of the linear heart tube, well after higher vertebrates specify SHF progenitors in the lateral plate mesoderm (LPM). Building on a recent dye tracing study from the Kirby laboratory, we identified putative nkx2.5+ SHF progenitors in the zebrafish anterior LPM (ALPM), cranial and medial to adjacent early-differentiating cardiomyocytes (nkx2.5+, cmlc2+). Using inducible Cre/loxP-mediated lineage tracing, we pulse-labeled nkx2.5+, GATA4+, or cmlc2+ cells in the ALPM and characterized their descendents using lineage specific reporters. From these analyses, we learned that pulse-labeled nkx2.5+ and GATA4+ cells give rise to myocardium in the entire ventricle and three cardiovascular li neages in the OFT. Pulse-labeling of cmlc2+ cells revealed that early differentiating cardiomyocytes reside predominantly in the proximal ventricle. Taken together, these data suggest strongly that nkx2.5+, GATA4+, cmlc2- SHF progenitors are specified in the zebrafish ALPM prior to initiating expression of ltbp3 at the arterial pole. Furthermore, we tested whether nkx2.5 plays a conserved and essential role during zebrafish SHF development. Nkx2.5 morphant embryos exhibited several SHF-related phenotypes including significant reductions in distal ventricular myocardium, outflow tract smooth muscle, and expression of ltbp3. Taken together, our data reveal two conserved features of zebrafish SHF development underscoring the utility of this model organism for deciphering SHF biology.

S7.3 Notch1 mediated signaling cascades regulate cardiomyocyte polarity and ventricular wall formation.

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Ventricular trabeculation and compaction are two anatomically overlapping morphogenetic events essential for normal myocardial development. Disregulation of these events can lead to Left Ventricular Noncompaction (LVNC, MIM300183). However, the underlying mechanisms and pathogenetic pathways remain elusive, largely due to the genetic heterogeneity of the patients and the lack of suitable animal models. Recently, through the use of gene profiling and cardiac cell-lineage restricted genetic manipulation, we analyzed a unique mouse genetic model for LVNC, *Fkbp1a* knockout mice. Our data demonstrated a critical contribution of over-activated Notch1 and subsequent neuregulin1-ErbB2/4 signaling in developing LVNC in mouse, and confirmed previous finding that Notch1-neuregulin1/Bmp10 signaling pathways in regulating ventricular cardiomyocyte proliferation. Remarkably, our new emerging data also suggested that the excessively activated Notch1-neuregulin1/ErbB2 signaling cascade dramatically down-regulated dishevelled-associated activator of morphogenesis 1 (Daam1), a member of the Formin family and a potential effector of non-canonical Wnt planer cell polarity (PCP) signaling. Genetic ablation of Daam1 resulted in disoriented cardiomyocyte polarity with altered actin cytoskeleton and myofiber organization, and concomitantly ventricular noncompaction, which confirmed the role of Daam1 in the development of LVNC. More interestingly, we have also identified that p21-activated kinase 1 (Pak1), a Ser/Thr protein kinase known to control tyrosine kinase receptor (*i.e.*, ErbB2/4) phosphorylation and small GTPases activities in regulating cell proliferation, cell polarity, and actin cytoskeleton organization, is a potential up-stream regulator of Daam1. Activated Pak1 (phospho-T423) was dramatically up-regulated in *Fkbp1a* mutant hearts with excessive activation of ErbB2/4. In addition, over-expression of constitutively active Pak1 (Pak1ca) in primary cultured neonatal cardiomyocytes down-regulated the level of Daam1. Taken together, our *in vivo* and *in vitro* data demonstrated that Notch-neuregulin-Pak1-Daam1 is an essential signaling cascade in regulating cardiomyocyte polarity and ventricular wall formation.

S7.4 Mutations in the NOTCH pathway regulator MIB1 cause ventricular non-compaction cardiomyopathy

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Ventricular non-compaction (VNC) cardiomyopathy patients show prominent trabeculations mainly in the left ventricle and reduced systolic function. Clinical presentation varies from asymptomatic to heart failure. We show that germline mutations in human MIND BOMB-1 (MIB1), encoding an E3 ubiquitin ligase that promotes endocytosis of the NOTCH ligands DELTA and JAGGED, cause VNC in autosomal-dominant pedigrees, and affected individuals show reduced NOTCH1 activity. Functional studies in cells and zebrafish embryos and in silico modeling indicate that these are loss-of-function phenotypes. VNC is triggered by targeted inactivation of Mib1 in mouse myocardium, and mimicked by inactivation of myocardial Jagged1 or endocardial Notch1. Myocardium-specific Mib1 mutants show reduced ventricular Notch1 activity, and an expansion of compact myocardium markers to the abnormally proliferative, immature trabeculae. These results identify NOTCH as a primary signaling pathway involved in VNC, and suggest that MIB1 mutations cause a developmental arrest in chamber myocardium, preventing trabecular maturation and compaction.

S7.5 The early role of *Tbx1* in anterior and posterior second heart field cells

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During cardiac development, the anterior second heart field (SHF) contributes cells to the arterial pole and the posterior SHF to the venous pole. T-box transcription factor Tbx1 is expressed in the SHF and is known to be involved in development of the arterial pole. Here, we aimed to elucidate the early role of Tbx1 in the posterior SHF and derived structures, and its relation to anterior SHF development. Using quantitative 3D reconstruction methodologies, we found that the proliferative rate and population size of SHF cells was severely affected in *Tbx1*-deficient mouse embryos. DiI labeling of anterior SHF cells in an *Fgf10* enhancer trap transgene revealed that, in the absence of *Tbx1,* the anterior movement of anterior SHF cells fails from the 7 somite stage. Moreover, these anterior SHF cells contribute to the venous pole and differentiate into atrial myocardium by activating the local atrial gene program. Distal hypoplasia of the outflow tract seems to be a late consequence of this defect. In addition, the distance between the outflow tract and the inflow tract was greatly reduced and the subsequent fusion of the dorsal mesocardium was consequently delayed. Our findings suggest that *Tbx1* is involved in maintaining the SHF population size from embryonic day (E) 8.0 onward and that *Tbx1*-deficiency disrupts proliferation, differentiation and cell fate decisions within the SHF during early heart morphogenesis. Moreover, despite prepatterning into anterior and posterior subdomains, cardiac progenitor cells of the SHF seem to be naïve in the absence of *Tbx1*.

S7.6 Retinol Dehydrogenase 10: Roles in Embryo Pharyngeal Patterning and a Model to Understand How Retinoid Gradients Prevent Congenital Birth Defects

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Retinoic acid (RA), an active vitamin A metabolite, is a key signaling molecule in vertebrate embryos. Morphogenetic RA gradients are thought to be set up by tissue-specific actions of retinaldehyde dehydrogenases (RALDHs) and catabolizing enzymes (CYP26s) and are important in preventing congenital malformations. The retinol to retinaldehyde conversion was believed to be achieved by several redundant enzymes; however, a random mutagenesis screen identified retinol dehydrogenase 10 (RDH10) as responsible for a homozygous lethal phenotype (Rdh10Trex mutants: Sandell et al., Genes Dev. 21, 1113-1124, 2007) with typical features of RA deficiency. We report the production and characterization of novel murine Rdh10 loss of function alleles generated by gene targeting. We show that, although the Rdh10-/- mutants die at an earlier stage than Rdh10Trex mutants, their molecular patterning defects do not reflect a complete state of RA deficiency. Genetic models of RA/RALDH2 deficiency p roduces hypoplastic development of the 3rd to 6th branchial arches, a malformation disrupting cardiac outflow tract septation. In Raldh2-/- mutants this defect cannot be restored by maternally administered RA. In Rdh10-/- mutants, pharyngeal growth and patterning is restored by administering retinaldehyde to pregnant mothers. We hence obtain viable Rdh10-/- mutants, free of lethal congenital malformations. This is the first demonstration of rescue of an embryonic lethal phenotype by simple maternal administration of the missing retinoid compound. These results underscore the importance of maternal retinoids in preventing congenital birth defects, and lead to a revised model on the enzymatic control of embryonic RA distribution.

7.7 Arid3b is required for heart development

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ARID3b is a transcription factor from the highly conserved ARID family, whose members have important roles in embryonic development and cancer. ARID3b-null mice die during early stages of embryonic development (E10.5) presenting severe defects in the cardiovascular system. However, its roles in development are not fully understood. Our aim is to address the function of Arid3b in the developing heart. We have analysed the expression pattern of this gene during mouse embryo development. Arid3b is expressed from early stages in the myocardium of the tubular hear and also in the cardiac precursors of the pharyngeal mesoderm. Later, its expression gets restricted to the second heart field derived poles of the heart. Using Arid3b deficient mice, we have analyzed the cardiac alterations produced by its absence. Three main defects can be highlighted: a noticeable shortening of the outflow tract; a reduction of the size of the inflow region with abnormal atria formation; and an alteration in the development of the atrio-ventricular canal (AVC), including defective formation of the AV cushions. Expression of several molecular markers of both secondary heart field and heart chambers is altered in mutant embryos. The defects found in mutant embryos and in vitro cell culture data support a hypothesis that Arid3b could be controlling the addition of precursor cells from the second heart field to the heart by regulating cell motility. Moreover, Arid3b seems to regulate the proper specification and differentiation of the myocardium in heart chambers versus the atrioventricular canal and to be involved in EMT.

7.8 Multiple influences of blood flow on cardiomyocyte hypertrophy in the embryonic zebrafish heart

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Cardiomyocyte hypertrophy is a complex cellular behavior involving coordination of cell size expansion and myofibril content increase. Here, we investigate the contribution of cardiomyocyte hypertrophy to cardiac chamber emergence, the process during which the primitive heart tube transforms into morphologically distinct chambers and increases its contractile strength. Focusing on the emergence of the zebrafish ventricle, we observed trends toward increased cell surface area and myofibril content. To examine the extent to which these trends reflect coordinated hypertrophy of individual ventricular cardiomyocytes, we developed a method for tracking cell surface area changes and myofibril dynamics in live embryos. Our data reveal a previously unappreciated heterogeneity of ventricular cardiomyocyte behavior during chamber emergence: although

cardiomyocyte hypertrophy was prevalent, many cells did not increase their surface area or myofibril content during the observed timeframe. Despite the heterogeneity of cell behavior, we often found hypertrophic cells neighboring each other. Next, we examined the impact of blood flow on the regulation of cardiomyocyte behavior during this phase of development. When blood flow through the ventricle was reduced, cell surface area expansion and myofibril content increase were both dampened, and the behavior of neighboring cells did not seem coordinated. Together, our studies suggest a model in which hemodynamic forces have multiple influences on cardiac chamber emergence: promoting both cardiomyocyte enlargement and myofibril maturation, enhancing the extent of cardiomyocyte hypertrophy, and facilitating the coordination of neighboring cell behaviors.

7.9 An essential role for H3K4 methyltransferase Mll2 in cardiac development

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During heart development, gene expression is tightly regulated by dynamic chromatin changes. H3K4 methylation is a histone modification that promotes transcriptional activation at associated gene loci. Mutations in *MLL2* (or *ALR*/ *KMT2D*), a H3K4 methyltransferase, leads to Kabuki syndrome and is often associated with congenital heart defects. Consequently, we hypothesize that H3K4 methylation is required during heart development, and Mll2 is a key methyltransferase involved in positively regulating cardiac gene expression. Early embryonic lethality of $Mll2^{\Delta/\Delta}$ mutants preclude assessment of its role in heart development. To determine the temporal requirement for Mll2 during cardiogenesis, we deleted *Mll2* in second heart field (SHF) progenitors (*Isl1::Cre*) and in differentiating ventricular myocytes (*Nkx2.5::Cre*). We observed embryonic lethality and ventricular septal defects (VSD) in both *Isl1::Cre* and *Nkx2.5::Cre* deletion mutants. However, $Isl1Cre;Mll2^{fl/fl}$ mutants also exhibited persistent truncus arteriosus (PTA) and thinned myocardium, suggesting a differential temporal requirement for Mll2 during heart development. Since Mll2 is not known to have intrinsic DNA binding specificity, we identified a candidate protein Ptip, a subunit unique to the Mll2 methyltransferase complex, which may recruit Mll2 to target genes. $Isl1::Cre;Ptip^{fl/fl}$ mutants had a similar phenotype to $Isl1::Cre;Mll2^{fl/fl}$ mutants, but $Nkx2.5::Cre;PTIP^{fl/fl}$ mutants were normal. These results suggest that Ptip may only be required for Mll2 recruitment at specific developmental stages. Overall, our work demonstrates that H3K4 methylation, as mediated by Mll2 and Ptip, is important for cardiac development. Future experiments will identify the cell types responsible for these cardiac defects and define the underlying molecular mechanism.

7.10 Regulation of myocardial proliferation in the developing embryo by Gata4 and Tbx5

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Gata4 and Tbx5 are key transcription factors that play central roles in heart development. *Gata4*;*Tbx5* compound heterozygote mice suffer early lethality and display complete atrioventricular septal defects, thin myocardium, and decreased myocardial proliferation, but the molecular mechanisms underlying this cardiac phenotype are unknown. To characterize this genetic interaction, the expression of Gata4 and Tbx5 expression during heart development was examined. Gata4 and Tbx5 are co-expressed in the atria and ventricle until E13.5, but afterward Tbx5 expression decreases in the ventricle. Consistent with this, thinning of the atrial and ventricular walls in compound heterozygotes is present in E11.5-E13.5 embryos. Gata4 and Tbx5 co-localize in atria and ventricle until E14.5, and subsequently co-localization is only maintained in the atria. In vivo co-immunoprecipitation data will be presented to demonstrate this interaction at various developmental timepoints. To determine the functional significance in vivo, we disrupted the myocardial interaction of Gata4 and Tbx5 by generating mice that were heterozygous for Gata4 in the myocardium and also haploinsufficient for Tbx5 ($Gata4^{flox/wt}$;αMHC-Cre^{+};$Tbx5^{+/-}$). These mice recapitulated the cardiac phenotype of Gata4;Tbx5 compound heterozygotes. The expression of Cdk2 and Cdk4, two genes important for cell cycle proliferation, was decreased in *Gata4*;*Tbx5* compound heterozygote hearts. The cardiac phenotype of *Cdk2* and *Cdk4* double knockout mice is similar to the *Gata4*;*Tbx5* compound heterozygotes, and Cdk4 has previously been demonstrated to be a direct target of Gata4 (Berthet, 2006; Rojas, 2008). Detailed analysis of the regulation of Cdk2 and Cdk4 by Gata4 and Tbx5 will be presented.

7.11 Mef2c Regulates Transcription of Cartilage Link Protein 1 in the Developing Heart

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Previous studies by the Wessels lab have demonstrated that Hyaluronan and Proteoglycan binding Link Protein 1 (Hapln1 or Crtl1/cartilage link protein) is involved in heart development. Crtl1/Hapln1 is an extracellular matrix (ECM) protein that stabilizes the interaction between hyaluronan and versican and is expressed in endocardial and endocardially derived cells in the developing heart, including cells in the atrioventricular (AV) and outflow tract (OFT) cushions. Hapln1/Crtl1 knockout mice have a range of cardiovascular malformations such as thin myocardium, atrioventricular septal defects (AVSD), and decreased trabeculation. Histological analysis of Hapln1/Crtl1 knockout mice reveals there is decreased expression of the Crtl1 binding-partners versican and that reduced expression of these ECM proteins may contribute to the cardiovascular malformations observed. Investigations into the transcriptional regulation of the Crtl1 gene have resulted in the finding that the cardiac transcription factor Mef2c binds to the Crtl1 promoter to regulate its expression in the endocardium and valve mesenchyme of the developing heart.

7.12 Shroom3 deficient mice show congenital heart defects

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Congenital heart defects (CHD) are associated with a number of genetic and environmental risk factors and remain one of the most common birth defects - affecting approximately 1-5% of newborns. Numerous transcription factors have been implicated in CHDs, however how their transcriptional activity relates to defects in morphogenesis remains to be explored. Shroom3 is an actin binding protein found to be essential for neural tube closure in mouse, *Xenopus* and chick by its ability to confer apical constriction. Shroom3 has also recently been associated with heterotaxy in human patients. Whole mount *in situ* hybridization for Shroom3 has revealed its expression within the forming heart of *Xenopus and loss of shroom3 activity results in malformed hearts*. Mice homozygous C57BL/6J Shroom3 gene trap insertion die at birth due to severe exencephaly but there is no information whether there are also cardiac defects. Here we provide evidence of multitude CHDs in the homozygous mutant mice. The homozygotes exhibit both atrial and ventricle septal defects as well as persistent hypertrophy of the ventricles, disorganized trabeculae and immaturity in the valves. Taking advantage of the lacZ gene trap, we have also characterized the expression of shroom3 during cardiogenesis. Our study is an initial step in characterizing the cell activities that drive cardiac morphogenesis and that result in CHDs when disrupted.

7.13 Function of Ddc in fetal and neonatal heart development.

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Ddc_exon1a is expressed solely from the paternally inherited allele in the developing and neonatal mouse heart, it is the only gene known to exhibit heart specific imprinting and is expressed from both parental alleles in all other tissues. *Ddc* is predominately expressed in the developing myocardium, which points to a role in fetal heart development. We have utilized knockout (DdcKO) mice, which harbour a germline deletion of *Ddc*, to examine its role in fetal heart development. Using microarray and episcopic fluorescence image capture technology (EFIC) we have examined heterozygous *Ddc* knockouts where the mutated allele has been inherited from the father for differential gene expression, gross cardiac phenotypes and embryonic growth perturbations. Our microarray results indicate that Ddc may play a role in cellular proliferation and morphogenesis of the developing myocardium by impacting the expression of around 20 genes involved specifically in ventricle formation. We also present EFIC data illustrating morphological differences between wild type and Ddc knockout hearts.

7.14 Roles of mesodermally-expressed integrin a5 during cardiovascular development

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Integrins are heterodimeric cell adhesion receptors. As major mediators of cell-ECM, integrins play important roles in cardiovascular development and other physiological and pathological processes. Integrin a5b1 acts as one of the main fibronectin (FN) receptors during embryogenesis, in part by binding to the RGD region of FN. Integrin a5 -null mouse embryos show very severe defects in cardiovascular development and die by embryonic day 10.5. Previously, we found that integrin a5 regulates the establishment of left-right asymmetry in mice and fish and plays an essential role in proliferation and survival of the cardiac neural crest-derived cells. These studies were conducted using global integrin a5-null mutants. In order to understand tissue-dependent roles of integrin a5 in embryonic patterning and neural crest development, we conditionally ablated integrin a5 in mesoderm using Mesp1Cre strain of mice. Descendants of Mesp1Cre-expressing cells give rise to the earliest-known cardiac progenitors, all endothelial progenitors and entire pharyngeal mesoderm. We found that conditional ablation of integrin a5 using Mesp1Cre leads to embryonic lethality by E16.5 due to severe defects in the morphogenesis of the cardiac outflow vasculature. In addition, majority of the mutants have defective thymus development, and some display aberrant development of the right accessory lobe of the lung. Defects in cardiovascular development included hemorrhage, double aortic arch, right sided aorta, left-sided aorta accompanied by the right sided ductus arteriosus, double outlet right ventricle, interrupted aortic arch, retroesophageal right subclavian artery, muscular ventricular septum defects and thin compact zone in the hearts of the mutants. Further in situ hybridization and immunohistochemistry studies are being conducted to determine the mechanisms underlying these morphogenetic defects. These findings will facilitate further understanding of the interactions between mesodermal cells and neural crest cells during cardiovascular development and the importance of integrin signaling in these processes.

7.15 Critical roles of *Mycn* during myocardial wall morphogenesis

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Our previous study revealed *Mycn* as a direct target of the BMP signaling pathway in developing hearts. The role of BMP signaling in regulating cardiogenesis has been well established. Furthermore, human genetic studies revealed that haploinsufficiency for *MYCN* causes Feingold syndrome, a developmental disease characterized in part by heart defects. In this study we test the hypothesis that myocardial *Mycn* is essential for normal myocardial wall morphogenesis through a conditional gene inactivation approach. Loss of myocardial *Mycn* caused embryonic lethality at midgestation. Mutants displayed severe hypoplastic myocardial walls, which is caused by both decreased cell proliferation and reduced cell size, but not by increased cell death. Expression of cell cycle regulatory genes was reduced in mutants. Furthermore, *Mycn* promotes cell growth through upregulating p70SK expression. Deletion of *Mycn* led to incomplete trabeculation, resembling mouse models with disruption in the Nrg-1/EphbB pathway. Treating embryonic hearts with an Ephrin-specific blocker severely reduced *Mycn* expression, suggesting that *Mycn* is a key target of Ephrin signaling in promoting trabeculation. Deletion of *Mycn* also eliminated NRG1 and BMP10 upregulated cell proliferation in cultured embryonic hearts, supporting its essential role in mediating activities of BMP and EGF signaling during cardiogenesis. Deletion of *Mycn* did not lead to pre-maturation of embryonic cardiomyocytes as evidenced from examining embryonic and adult specific cardiomyocyte markers. In conclusion, our study reveals *Mycn* as a key transcription factor mediating activities of multiple signaling pathways in promoting cardiomyocyte proliferation and myocardial wall morphogenesis.

7.16 Cyp26 enzymes are required during two distinct phases for proper patterning of zebrafish heart chambers

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Normal heart development requires appropriate levels of retinoic acid (RA) signaling, with too much or little being teratogenic. One way the amount of RA is moderated in the embryo is by Cyp26 enzymes, which metabolize RA into easily degraded derivatives. However, the role Cyp26 enzymes play during heart development has not yet been addressed. In zebrafish, we found that two Cyp26 enzymes, Cyp26a1 and

Cyp26c1, are expressed in the anterior lateral plate mesoderm marginally overlapping with the early cardiac progenitors. While singular knockdown of Cyp26a1 or Cyp26c1 does not overtly affect heart development, the hearts of the Cyp26a1 and Cyp26c1 deficient embryos are linearized with smaller ventricles and dilated atria by 48 hours post fertilization (hpf). Interestingly, the phenotype observed in double morphants does not replicate the teratogenic effects seen when embryos are treated with high concentrations of RA, which can eliminate cardiomyocytes. Closer examination of when cardiomyocytes are affected revealed two phases of Cyp26 enzyme requirement. We find an earlier role where loss of Cyp26 enzymes leads to increased atrial cardiomyocyte differentiation, which translates into more atrial cells at 48 hpf. Although we do not find an earlier effect on ventricular cardiomyocyte differentiation, we find ventricle cardiomyocyte number is reduced by 48 hpf and progressively lost through 72 hpf. Therefore, our results suggest a previously unappreciated model where Cyp26 enzymes are required to prevent increases in RA signaling at two distinct phases to allow for proper heart development.

7.17 Development of atrial septum: Chick vs Mouse

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Six different cell populations participate in atrial septation: the atrial mesenchymal cap (AMC); dorsal mesenchymal protrusion (DMP); mesenchyme from the superior and inferior cushions of the AV canal; a myocardial cell population from the septum primum (SI); and from the septum secundum (SII). In placental mammals those cell populations are integrated during the three phases of atrial septation: Phase I, is initiated by the formation of the AMC, which together with the inferior cushion, superior cushion and by the DMP, encircled the ostium primum. Over the SI openings are formed that coalescent to form the ostium secundum; and the ostium primum is closed by the fusion of the AMC with the inferior cushion. Phase II, includes the formation of the SII which grows ventrally until it forms a semilunar-shaped structure (Foramen Ovale). Phase III, the SI and SII fuse to form the definitive atrial septum. It is important to mention that avian and lower mammals only present a Phase I. The Periostin expression correlates with the transformation of the muscular SI into mesenchyme, and its expression is maintained until the closure of ostium II. We report on the development of chick culture model to study the molecular mechanisms (periostin initially) that govern the Phase I of atrial septation that is extendable to the mouse. Supported by: 2R01HL033756-27; Leducq (07CVD04); COBRE (P20 RR016434/P20 RR021949-01A2).

7.18 IGF2 Signaling Regulates Cardiomyocyte Proliferation

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Our recent results show that IGF2 is the major epicardial mitogen that supports embryonic ventricular cardiomyocyte proliferation. For this analysis, we employed cardiac-specific (Nkx2.5Cre) IGF receptor mutants and global Igf2 ligand mutant embryos. We have now crossed Nkx2.5Cre with a conditional Igf2 allele and confirmed that the source of the IGF2 ligand is within the heart (within the Nkx2.5Cre recombination domain). We are now in the process of crossing the conditional Igf2 allele with epicardial-specific as well as endocardial- and myocardial-specific Cre lines to further define the source of this ligand. Embryos globally lacking the retinoic acid (RA) receptor RXRa are also compromised in cardiomyocyte proliferation and heart development. Epicardial Igf2 expression is diminished in RXRa mutants, linking these two genes in a common pathway; however, the Igf2 gene is not directly activated by RA. We crossed a number of Cre lines and found mesodermal-specific Mesp1Cre o r neural crest cell lineage-specific Wnt1Cre did not replicate the phenotype of global RXRa deficiency. However, the more broadly active mesodermal Brachyury (T)-Cre impacted heart development. We had previously shown that RA acts in the fetal liver to directly control expression of the cytokine erythropoietin (EPO). T-Cre is active in the fetal liver, whereas Mesp1Cre is inefficient in liver recombination. We found that EPO addition rescued Igf2 expression in RA-deficient embryonic hearts. Our results support a model in which RA signaling in mesodermal cells of the fetal liver supports Epo expression, which then influences epicardial Igf2 expression and subsequent cardiomyocyte proliferation.

7.19 Roles of LRRC10, a cardiac specific factor in heart development and function

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Leucine Rich Repeat Containing protein 10 (LRRC10) has been identified as a cardiac-specific factor. Knockdown of *lrrc10* in zebrafish embryos caused severe cardiac morphogenic defects including a cardiac looping failure and a decrease in cardiac output, resulting in embryonic lethality. Since the roles of LRRC10 in mammalian hearts remain to be elucidated, *Lrrc10* knock out (KO) mice were investigated. *Lrrc10* KO mice exhibit systolic dysfunction in utero and develop dilated cardiomyopathy in early postnatal life. LRRC10 is detected both in cytoplasm and nuclei of embryonic cardiomyocytes but shows striations in adult cardiomyocytes. Gene expression profiling experiments identified multiple defective molecular pathways both in embryonic and adult *Lrrc10* KO hearts, but revealed distinct molecular defects in pre *vs* post natal hearts. Pathway analysis identified regulation of the actin cytoskeleton as a significantly upregulated pathway in embryonic *Lrrc10* KO hearts and signal transduction pathways involving AKT and PKCε are altered in adult *Lrrc10* KO hearts. Yeast two-hybrid screening and coimmununoprecipitation studies indicate that LRRC10 interacts with α-actinin and actin isoforms, implying that LRRC10 serves as a mechanical link between the myofilament Z-disc and the cytoskeleton. Our study demonstrates that the developmental perturbation of cardiac biology in the absence of LRRC10 results in adult heart disease. The precise molecular function of LRRC10 is being investigated, which will enhance our understanding of congenital heart defects and heart disease. In summary, we identify LRRC10 as a gene crucial for embryonic cardiac function and proper development and a novel dilated cardiomyopathy candidate gene.

7.20 Signalling via Bmp Type I receptor Alk2 (Acvrl) in second heart field cells and/or endocardium is required for right ventricle and outflow tract development

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Bmps are required for normal development of right ventricle, outflow tract and great vessels. Using the *MEF2c-AHD-Cre* driver, we are investigating the role of Bmp signaling through Alk2. Post-septation conditional *Alk2*-null embryos show a range of OFT/truncal phenotypes, from double outlet right ventricle to common arterial trunk, and abnormal embryonic RV morphology is recognizable from E9. A number of studies have suggested that the balance between Bmp and Fgf signaling, and Bmp-induced miRNAs, play key roles in this process. Using Q-RTPCR, we examined expression of key genes in RV+OFT at E9 and, using *R26R-YFP* and FACS sorting, in recombined SHF cells outside the heart at E9 and at E10. Expression of Bmp signaling target *Smad6* was reduced in mutants in all three tissues. Although *Isl1* was increased in RV+OFT in *Alk2* mutant RV+OFTs, *Fgf10* showed no consistent difference. *Tbx1* expression was inconsistently elevated in E9 SHF, but *Isl1* and *Fgf10* were not different from controls. Levels of *miRNA17* and *20a* did not differ between *Alk2* mutants and controls in either dissected E9 SHF-enriched tissues or E9 hearts. These results suggest that the altered expression of genes regulated by Bmp signaling happen at different times/in different populations of cells, and that *miRNA17* and *20a* do not play a major role in our *Alk2* cko model. We have detected *Alk2* in SHF cells by WMT ISH and using an *Alk2* reporter BAC line at E8, showing that *MEF2c-AHD-Cre* recombination in these cells could directly underly at least the earliest abnormal phenotype.

7.21 WNT/β-catenin Signaling Promotes Growth of Ventricular Myocytes from the First- and Second-Heart-Field

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Elucidating the pathways that control ventricular growth and development is key to developing novel approaches in regenerative cardiovascular therapy. Cells from the first- and second-heart-field give rise to distinct regions of the four-chambered heart. Previously it is shown that canonical WNT signaling has bimodal effects on differentiation and growth of the developing heart fields (E7.0-9.5) and in the early heart (E9.5-12.5). However it remains unclear what the role of WNT signaling is in later stages of cardiac development. We asked the specific question of what role canonical WNT signaling plays in late ventricular development. Therefore we used an *in*

vitro double transgenic mouse reporter system to isolate ventricular progenitors of the first- and second-heart-field. In these, we overexpressed WNT/β-catenin signaling by a small molecule known to target this pathway. Upon overregulation of WNT we found a significantly higher proportion of proliferating ventricular progenitors and myocytes in both populations. We also looked at the function of canonical WNT signaling in controlling heart growth *in vivo*. Therefore we stabilized β -catenin in ventricular MYL2 expressing cells using a Cre-LoxP recombination. At P1 we found an increase in proliferation of left and right ventricular myocytes. Preliminary data shows that P35 mice with MYL2 WNT overexpression have a modest increase in heart size. Altogether these data suggest that WNT/β-catenin signaling is important in increasing late embryonic and postnatal ventricular myocyte growth and thereby heart size control.

7.22 BMP Signaling and Atrioventricular Septation

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Atrioventricular septal defects (AVSDs) encompass a spectrum of malformations in which deficient septation permits the chambers of the heart to communicate. These defects are relatively common; they comprise 3-5% of all congenital cardiac malformations and are present in approximately 25% of children with Down syndrome. For many years, AVSDs were thought to arise solely from malformation of the endocardial cushions. More recently, however, defective development of the dorsal mesenchymal protrusion (DMP), a derivative of the Second Heart Field, has also been implicated in the pathogenesis of AVSDs. Insight into the mechanism(s) governing proper development of the DMP is slowly emerging. In this study we present data demonstrating that BMP signaling is critical for DMP formation and/or maturation. We show that pSMAD1/5/8 is expressed at high levels in the DMP precursor population, that BMP ligands are expressed around the developing DMP, and that deletion of the BMP type I receptor ALK3 from the DMP precursors results in a hypoplastic DMP and a fully penetrant AVSD phenotype. While the importance of BMPs in the development of the outflow tract and AV junction is well established, our work indicates a novel role for BMP signaling at the venous pole of the heart.

7.23 Hedgehog-dependent atrial septum progenitors are required for cardiac septation

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Atrial septation is a critical step in separating the systemic and pulmonary circulations in tetrapods and atrial septal defects are among the most common forms of human congenital heart disease. We have previously demonstrated that the atrial septum is generated by second heart field (SHF) progenitors that receive Hedgehog (Hh) signaling (Hoffmann et al., 2009). To identify Hh-dependent pathways for atrial septation, we performed transcriptional profiling on SHF tissues of wild-type and *shh* knockout mice. We have identified and verified genes whose SHF expression is Hh- dependent by RT-PCR and examined several genes by in-situ hybridization. Interestingly, the expression of Hh-dependent transcripts cluster into distinct SHF domains: (1) genes implicated in proliferation in other contexts, such as FGF8, are expressed in an arch-shaped pattern lateral to and surrounding the dorsal mesocardium and (2) genes implicated in differentiation in other contexts, such as BMP4, appear to be located more medial and directly adjacent to the dorsal mesocardium. We have directly examined the requirement for these pathways in atrial septation and find that removal of FGF8, FGFR1 and 2, or BMP4 from SHF tissues results in failure of atrial septation. We are currently testing the domain hypothesis that laterally-expressed genes control posterior SHF proliferation, while medially-expressed genes trigger differentiation. These results form the basis for investigating the cellular and molecular mechanisms required for generation of the atrial septum from SHF cardiac progenitors.

7.24 The Planar Cell Polarity Signaling Pathway Is Required In The Second Heart Field Lineage For Outflow Tract Morphogenesis

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Outflow tract (OFT) malformations underlie a majority of human congenital heart defects. The OFT arises in part from the Second Heart Field (SHF) progenitors in the pharyngeal and splanchnic mesoderm (SpM), outside of the initial heart. While SHF proliferation and differentiation have been extensively studied; how these progenitors are deployed to the OFT remains unclear. Using a set of mouse *Dishevelled2 (Dvl2)* alleles, we demonstrate

that the planar cell polarity (PCP) pathway, a branch of the β-catenin independent non-canonical Wnt pathway that regulates cellular polarity and polarized cell behavior during tissue morphogenesis, is required specifically in the SHF for early OFT morphogenesis and may play a key role in SHF deployment. We find that mutations in mouse core PCP genes *Dvl1/2* and *Vangl2* as well as non-canonical Wnt gene *Wnt5a* result in aberrant cardiac looping. The looping defect is correlated with severe OFT shortening at embryonic day 9.5, characteristic of comprised contribution from the SHF. Consistent with our genetic interaction studies suggesting that Wnt5a signals through the PCP pathway, *Dvl1/2* and *Wnt5a* mutants display aberrant cell packing along with diminished actin polymerization and filopodia formation in SHF progenitors in the caudal SpM, where Wnt5a and Dvl2 are co-expressed specifically. Based on these results, we propose a novel model in which a Wnt5a→Dvl PCP signaling cascade promotes SHF deployment by promoting actin polymerization and protrusive cell behavior to continuously recruit SHF progenitors into a cohesive sheet in the caudal SpM, thereby pushing the SpM rostrally into the OFT.

7.25 Casz1 is Required for Cardiac Development

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Understanding the molecular mechanisms of cardiomyocyte cell fate decisions is critical for uncovering pathologies and treatments for congenital heart disease. The program of differentiation and the identity of the molecules and signals that regulate the fate of cardiac progenitor cells and their ability to differentiate into the major functional cell lineages of the heart remain important areas of study. Here we establish that the zinc finger transcription factor Castor (Casz1) displays an evolutionarily conserved expression pattern in cardiac tissue. We have gone on to generate a conditional allele of Casz1 and demonstrate that the cardiac-specific ablation of Casz1 leads to embryonic lethality with homozygous null mice exhibiting a range of cardiac malformations including ventricular septal defects. To extend and complement these studies, we sought to determine the fate of Casz1 cells in the developing embryo. To this end, we have generated lines of mice in which we have knocked-in an inducible CreERT cassette into the Casz1 locus (Casz1KI-CreERT2). Results of fate mapping studies using the Casz1KI-CreERT2 allele demonstrate that Casz1-expressing cells give rise to cardiac structures including the atria and interventricular septum. Collectively, these studies demonstrate that Casz1 is essential for cardiac development and has an essential role in septum formation.

Section 8: Valve Development

S8.1 Computational modeling of endocardial cell activation during AV valve formation.

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The development of valve structures occurs in distinguishable phases, which are highly conserved. The first event during valve development is the induction of endocardial cushions (ECs) within the atrioventricular (AV) canal and outflow tract of the primitive heart tube. It has been well established that a BMP signal from the myocardium initiates this event by the induction of TGF-β, *Has2* (hyaluronic acid synthase 2), and Notch1, as well as the transcription factors Snail1 and Twist1. The importance of regulating *Has2* expression in the endocardium has been exemplified by the observation that in *Has2* deficient mice or zebrafish the cardiac jelly does not expand and ECs fail to form. We recently showed that zebrafish *dicer* mutant embryos, lacking mature miRNAs, form excessive endocardial cushions (Lagendijk et al. *Circ Res*. 2011). By functional screening we found that miR-23 is both necessary and sufficient for restricting the number of activated endocardial cells in the ECs. In addition, in mouse endothelial cells, miR-23 inhibited a TGF-β–induced endothelial-to-mesenchymal transition. By *in silico* screening combined with in vivo testing, we identified *Has2*, *Icat,* and *Tmem2* as novel direct targets of miR-23. Surprisingly, miR-23 expression is confined to the ECs where its activity is required to repress Has2. To understand the significance of the miR-23-Has2 interaction we applied computational modeling using a cellular Potts algorithm. The computational model predicts a regulatory network of both positive and negative feedback mechanisms to restrict the activation of the endocardial cells to the AV canal.

S8.2 Tie1 is Required for Semilunar Valve Form and Function

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The mechanisms regulating late gestational and early postnatal semilunar valve remodeling and maturation are poorly understood. Tie1 is a receptor tyrosine kinase with broad expression in embryonic endothelium. During semilunar valve development, Tie1 expression becomes restricted to the turbulent, arterial surfaces of the valves in the perinatal period. Previous studies in our laboratory have demonstrated that Tie1 can regulate cellular responses to blood flow and shear stress. We hypothesized that Tie1 signaling would regulate the flow dependent remodeling of the semilunar valves associated with the conversion from maternal/placental to independent neonatal circulation. To circumvent the embryonic lethality, we developed a floxed Tie1 allele and crossed it to an Nfatc1 P2-Cre line that mediates gene excision exclusively in the endocardial cushion endothelium. Excision of Tie1 resulted in aortic valve leaflets displaying hypertrophy with perturbed matrix deposition and re modeling without alteration in cell number, proliferation, or apoptosis. Differences were only detected after birth, increased with age, and were restricted to the aortic valve. RNA sequence analysis suggests that mutant aortic valves are similar to wildtype pulmonary valves in transcriptional profile. The aortic valves demonstrated insufficiency and stenosis by ultrasound and atomic force microcopy documented decreased stiffness in the mutant aortic valve consistent with an increased glycosaminoglycan to collagen ratio. These data suggest that active endocardial to mesenchymal signaling, at least partially mediated by Tie1, is uniquely required for normal remodeling of the aortic but not pulmonary valve in the late gestation and post-natal animal. Suppt. by RL1HL0952551 (HSB), HL094707 (WDM), HL078881 (BZ).

S8.3 In Vivo Reduction Of Smad2 Concomitant With Proteoglycan Accumulation Results In High Penetrance Of Bicuspid Aortic Valves

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Bicuspid aortic valve (BAV) is the most prevalent cardiovascular malformation resulting in two rather than three mature cusps and occurs in at least 1-2% of the population. However, only NOTCH-1 loss of function has been linked to human BAV disease, and there are limited genetically modified mouse models that display BAV. We reported that loss of proteolytic cleavage of versican, a proteoglycan abundant in cardiac outlet cushions, results in myxomatous valves in ADAMTS5 deficient mice. Recently we discovered that a failure to cleave versican was concomitant with a reduction of cell-cell condensation, phosphorylated Smad2 and fibrous ECM organization in *Adamts5*$^{-/-}$ valves. To test the hypothesis that that versican cleavage via ADAMTS5 is required to reduce the versican-hyaluronan pericellular matrix to elicit Smad2 phosphorylation we further reduced Smad2 in *Adamts5*$^{-/-}$ mice through intergenetic cross. The resulting *Adamts5*$^{-/-}$*;Smad2*$^{+/-}$ mice developed a more dramatic valve phenotype than *Adamts5*$^{-/-;}$*Smad2*$^{+/+}$ mice and displayed a high penetrance of BAV (6/9). Interestingly, the pulmonary valve was also bicuspid in 5/9 (4/9 displayed both bicuspid PV and BAV). All *Adamts5*$^{-/-}$*;Smad2*$^{+/-}$ semilunar valves displayed wide hinge regions at the juncture of the annulus concomitant with loss of fibrous ECM. These data suggest that the dramatic changes in proteoglycan turnover, during remodeling of the truncal cushions, elicit changes in cell behavior and signaling required for normal semilunar cusp formation. Further studies of the *Adamts5*$^{-/-}$*;Smad2*$^{+/-}$ mice may elucidate a novel etiology of BAV pathogenesis and lead to new pharmacological treatments for valve disease.

S8.4 Filamin-A Regulates Tissue Remodeling of Developing Cardiac Valves via a Novel Serotonin Pathway

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Linkage and sequencing studies of patients with myxomatous valvular dystrophy revealed causal mutations in the Filamin-A gene. These studies defined Filamin-A as integral to valve structure and function. However, the

mechanisms by which Filamin-A regulates valve cell biology are unknown. Herein we demonstrate that Filamin-A conditional KO (cKO) mice exhibit enlarged leaflets commencing during fetal valve maturation. This defect is caused by an inability for valve fibroblasts to efficiently remodel their extracellular matrix environment. Using the patient mutations as a guide, we identified a novel mechanism by which cooperative interaction between intracellular serotonin, transglutaminase-2 (TG2), and Filamin-A are required for promoting cytoskeletal-driven matrix remodeling events. Co-expression of serotonin, serotonin transporter (SERT), TG2, and Filamin-A is restricted to fetal valve development during active matrix remodeling. At this timepoint, immunoprecipitation experiments demonstrate that serotonin is covalently bound to Filamin-A *in vivo* and *in vitro* and is dependent on intact TG2 activity. Pharmacological and genetic perturbations of this interaction demonstrate serotonin transport inhibition, abrogation of TG2 activity, and/or genetic removal of Filamin-A result in loss of serotonin-Filamin-A interaction and impaired matrix remodeling. These findings illustrate a novel mechanism by which intracellular serotonin, TG2 and Filamin-A cooperatively regulate cytoskeletal-driven matrix remodeling. As such, these data provide mechanistic insight into normal processes driving cardiac valve maturation with added potential for providing insight into the developmental basis for human degenerative cardiac valve disease.

S8.5 Epicardially-derived Fibroblasts Preferentially Contribute to the Parietal Leaflets of the Atrioventricular Valves in the Murine Heart

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The importance of the epicardium for valvuloseptal development has been well established. To determine whether and if so how epicardially derived cells contribute to the developing valves in the murine heart we used a mWt1/IRES/GFP-Cre mouse to trace the fate of EPDCs from embryonic day (ED)10 until 4 months of age. Migration of EPDCs into the atrioventricular cushion mesenchyme starts around ED12. As development progresses, the number of EPDCs in the cushions increases significantly, specifically in the leaflets which derive from the lateral atrioventricular cushions. In these leaflets, the epicardially-derived fibroblasts eventually largely replace the endocardially-derived cells. Importantly, the contribution of EPDCs to the leaflets derived from the major AV cushions is very limited. The differential contribution of EPDCs to the respective leaflets of the atrioventricular valves provides a new paradigm in valve development and could lead to new insights into the pathogenesis of abnormalities that preferentially affect individual components of this region of the heart. The notion that there is a significant difference in the contribution of epicardially and endocardially derived cells to the individual leaflets of the atrioventricular valves has also important pragmatic consequences for the use of endocardial and epicardial cre-mouse models in heart development.

S8.6 Endothelial nitric oxide signaling regulates Notch1 in aortic valve development and disease

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Aortic valve calcification is the third leading cause of heart disease in adults and is frequently associated with bicuspid aortic valve, the most common type of cardiac malformation. NOTCH1 and the nitric oxide (NO) signaling pathway have been implicated in aortic valve development and calcification. Using an established porcine aortic valve interstitial cell (PAVIC) culture system known to spontaneously calcify, we demonstrate that gain or loss of NO, secreted by endothelial cells, prevents or accelerates calcification of PAVICs, respectively. Overexpression of Notch1 prevented the calcification that occurred with inhibition of NO, demonstrating the effects of NO on calcification are mediated by Notch1. Furthermore, endothelial cells or addition of NO regulates the nuclear localization of Notch1 and affects the expression of Hey1, a Notch1 target gene in PAVICs. To determine if Notch1 and endothelial nitric oxide (eNOS) genetically interact *in vivo*, we generated $eNOS^{-/-};Notch1^{+/-}$ compound mutant mice. Embryonic examination revealed cardiac abnormalities in a subset of $eNOS^{-/-};Notch1^{+/-}$ mice, which suffered ~70% lethality by postnatal day 10. Surviving compound mutant mice at 8 weeks of age demonstrated severely malformed aortic valves. $eNOS^{-/-};Notch1^{+/-}$ mice maintained on high fat diet for 12 weeks displayed aortic regurgitation and stenosis, thickened aortic cusps and development of calcific nodules on the aortic valve leaflets as compared to age-matched $eNOS^{-/-}$ mice. Overall, these data

demonstrate a novel molecular pathway by which endothelial nitric oxide regulates Notch1 signaling in a manner that is critical for aortic valve development and disease.

8.7 Analysis of TGFβ3 function in valve remodeling and aortic valve calcification

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Transforming Growth Factor beta (TGFβ) ligands function during the onset and progression of aortic valve calcification remains obscure as both contradictory elevated and suppressed TGFβ signaling has been found in patients of calcific aortic valve disease (CAVD). Here, using in vitro micromass cultures of mouse valve precursor cells (tsA58-AVM) we showed by complimentary realtime QPCR and synthetic luciferase reporter assays that TGFβ3 can upregulate Sry-related high-mobility-group box 4 (SOX4) transcription. Sox4 is initially expressed within the endocardially-derived tissue of both the outflow tract and atrioventricular canal during development, but persists within adult valve mesenchyme. Moreover, TGFβ3-dependent regulation of Sox4 can be blocked by a small molecule inhibitor of SMAD3 (SIS3), indicating that SMAD3 acts as a downstream mediator of TGFβ3-dependent Sox4 induction. Significantly, in situ hybridization revealed that TGFβ3 is the most prevalent TGFβ isoform in postnatal heart valves. Histology demonstrated that both$Tgfb3^{-/-}$ fetal (E14.5) and $Smad3^{-/-}$ adult (6 month old) valves were abnormally thickened. There was no calcification in the aortic valves of $Smad3^{-/-}$mice. Correlative analysis of human valve tissues via immunohistochemistry (5 controls, 8 CAVD, 4 non-calcific aortic valve disease) demonstrated diffused expression of SOX4 in the control tissues, which was extinguished in the diseased tissue specimens. Interestingly, the expression of SOX4 around the calcific nodules remains upregulated within the diseased aortic valves from CAVD patients. Consistently, Sox4 siRNA knockdown in tsA58-AVM cells significantly decreased calcification. Overall, our results indicate that TGFβ3-dependent and SMAD3-mediated regulation of SOX4 may play an important role in valve remodeling and aortic valve calcification. Since small molecule inhibitor/s of SMAD3 could be used as a therapeutic approach, these data provide novel basic science rationale with potential to translate into different therapies for patients of CAVD.

8.8 Nuclear exclusion of Sox9 in valve interstitial cells is associated with calcific phenotypes in heart valves

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Calcific aortic valve disease (CAVD) is a major public health problem with no effective treatment available other than valve replacement surgery. Despite the clinical significance, the mechanisms of pathogenesis are poorly understood. Normal valve structures are composed of organized layers of extracellular matrix interspersed with valve interstitial cells (VICs) that share molecular phenotypes with cartilage, including Col2a1. In contrast, calcified valves are characterized by mineralized matrix and increased expression of osteogenic genes including Spp1. We have shown that Sox9 is required in vivo to promote the cartilaginous matrix and prevent calcification of healthy valves by directly activating Col2a1 and suppressing Spp1 in the nucleus. In this study, we show that in aortic valves from non-diseased subjects, Sox9 expression is restricted to the nuclei of VICs, while nuclear expression is downregulated and notably cytoplasmic in tissue collected from CAVD patients. Usin g a pharmacological approach, we show that Sox9-mediated calcification as a result of nuclear exclusion can be induced by retinoic acid (RA) treatment in vivo and in vitro. This decrease in nuclear Sox9 localization is associated with decreased Col2a1 expression and transactivation, activation of the Gene Ontology 'bone development' program and matrix mineralization. Ongoing studies include defining the signaling pathways that maintain Sox9 nuclear localization to prevent CAVD in healthy valves. Together, these data suggest that Sox9 nucleocytoplasmic shuttling of Sox9 plays a role in valve pathogenesis and provides insights to aid in the development of new therapeutic strategies for CAVD.

8.9 Runx2 isoforms have divergent roles in development and pathology in the heart

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Formation of atrioventricular (AV) valves in the heart begins with an epithelial-mesenchymal cell transition (EMT). In the exploration of EMT, we investigated the presence of the transcription factor, Runx2, in the AV canal. Immunostaining and PCR showed that expression coincided with the onset of EMT. siRNA treatment of AV canal cultures established that it was required for EMT and that loss specifically prevented the early cell separation step of EMT. Sequence analysis showed that the Runx2 isoform present was Runx2-I and that this isoform was regulated buy TGFβ2 but not BMP. Inhibition of Runx2-I with siRNA produced a loss of mesenchymal cell markers. Comparison with siRNA towards Snail2, suggests that the two EMT transcription factors diverge in their regulation of downstream components. To determine whether the activity of Runx2-I was conserved in other EMT events, we examined human tissue samples in the progression of esophageal adenocarcinoma. Runx2-I was e xpressed coincident with the transition from columnar epithelia (Barrett's Esophagus) to dysplasia, suggesting a conserved role in early EMT. The Runx2-II isoform is associated with bone and calcifying tissues. To compare isoform activities, we examined Runx2 isoform expression in whole valve cultures from chicks and in micromass cultures of mouse valvular mesenchymal cells. Runx2-II inceases in culture consistent with expression of calcification markers including osteopontin and matrix gla protein and with positive staining for calcium. Together, the data show a requirement for TGFβ-regulated Runx2-I in EMT and BMP-regulated Runx2-II with calcification and calcified tissues. Supported by NHLBI HL82851 and Edwards Life Sciences.

8.10 3D cardiac valve tissue reconstruction and quantitative evaluations of tissue & cell phenotypes in the Pdlim7 knock-out mouse.

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The actin binding protein Pdlim7 is part of a family of proteins involved in regulating actin dynamics. Pdlim7 is expressed throughout cardiac development and into adulthood, and inactivation in zebrafish and mouse models results in cardiac valve malformations. A recent model for atrio-ventricular (AV) valve formation and maturation describes the differential contribution of epicardially- and endocardially-derived cells to specific leaflets of the mouse valvuloseptal complex. Using the Pdlim7 knock-out mouse, we investigated whether the aberrant morphology of the adult mitral valve is caused by the distinct lineages that shape the lateral and septal leaflets. We integrate 3D heart valve reconstructions with novel methods to quantitatively test morphological differences between wildtypes and mutants. The quantitative analysis of valve shape demonstrates that our method can identify subtle, statistically significant morphological changes following loss of Pdlim7. From Pdlim7's role in actin dynamics, it is possible that the valve tissue difference is the result of aberrant cellular migration and/or cellular shape. To investigate this, we present a 3D method developed to measure cell migration behavior, and introduce the field of 3D geometric morphometrics to studies of morphogenesis and statistically evaluate the patterns of cell shape diversity. This work in mice suggests Pdlim7 functions in AV valve remodeling and maturation at late embryonic and postnatal stages corresponding with the arrival of cells from the epicardial lineage. These findings are important because they describe a mouse model that links a novel gene (Pdlim7) to post-natal valve maturation and growth.

8.11 Elastic Fiber Defects In Emilin Deficient Aortic Valves Result In Early Bmp-Dependent Angiogenesis And Late Tgfβ-Dependent Fibrosis

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Emilin-1 (Elastin Microfibril Interface-Located protein) is an elastin binding protein that regulates elastogenesis and inhibits TGF-β signaling. Emilin-1 is expressed in the developing and mature heart valves, and Emilin-1 deficiency results in elastic fiber assembly defects. Aortic valve disease (AVD) is characterized by elastic fiber fragmentation, fibrosis and aberrant angiogenesis. We hypothesized that dysregulation of canonical and non-canonical TGF-β signaling induces fibrosis and angiogenesis respectively in the Emilin-1 deficient (Emi1−/−) model of AVD. The expression of Emilin-1 binding proteins elastin and fibulin-5 (an angiostatic factor) was decreased at the adult and aged stages. In addition, the Emi1−/− aortic valve displays a marked increase in

angiogenic factors (VEGF) at the juvenile stage and fibrosis at the aged stage only. Interestingly, pSmad1/5/8, p-Erk1/2, and elastase expression were increased at early stages, while p Smad2 and macrophage expression was increased at the late stage only. Although aged Emi1−/− valve tissue did not show calcification, cultured Emil1−/− valve interstitial cells do calcify faster and more abundantly than control cells in response to osteogenic stimuli. Animals die prematurely at the age of 14-18 months. Echocardiography showed normal ventricular function in Emil1−/− adults, and marked ventricular dysfunction in aged mutants. These findings identify the Emil1−/− mouse as a model of latent progressive AVD, similar to human disease, and implicate early BMP signaling dysregulation in the angiogenic response and late TGF-β signaling dysregulation in the fibrocalcific and inflammatory responses.

8.12 A Spatial Transcriptional Profile of the Chick and Mouse Endocardial Cushions Identify Novel Regulators of Endocardial EMT *in vitro*

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Early in valvulogenesis a subpopulation of endocardial cells overlaying the cushions of the atrial-ventricular canal (AVC) and outflow tract (OFT) undergoes an epithelial-to-mesenchymal transformation (EMT). We developed a strategy to identify genes important in endocardial EMT using a spatial transcriptional profile. AVC, OFT, and ventricles (VEN) were isolated from chick and mouse embryos at comparable stages of development (chick HH18; mouse E11.0) when EMT occurs in the AVC and OFT, but not VEN. RNA sequencing analysis of gene expression between these three regions was performed. 347 genes in the chick (n=1) and 225 genes in the mouse (n=2) were enriched 1.25 fold in the cushions. 18.2% mouse cushion enriched genes (41/225) had known roles in valve development. Gene ontology (GO) analysis of these gene lists revealed biological processes associated with EMT (cell adhesion, cell movement, ECM organization) that were conserved between chick and mouse. Gene regulatory networks (GRN) generated from cushion enriched gene lists identified TGFb and BMP as nodal points. Five candidate genes whose role in endocardial EMT *in vitro* was unknown, HAPLN1, ID1, FOXP2, TPBG, and MEIS2, were selected for analysis in an *in vitro* collagen gel assay. Knockdown of each resulted in an inhibition of EMT, functionally validating these genes as regulators of EMT *in vitro.* Our spatial transcriptional profiling strategy not only yielded gene lists which reflected the known biology of the system, by GO and GRN analysis, but accurately identified and validated novel candidate genes with previously undescribed roles in endocardial cell EMT *in vitro*.

8.13 Periostin/Filamin A: a candidate central regulatory mechanism for valve fibrogenesis and matrix compaction.

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We have previously shown that periostin (PN), a matricellular protein secreted by prevalvular cushion mesenchyme promotes cell autonomous, fibrogenic differentiation and compaction of collagenous matrix into mature valve leaflets and cusps. Loss of periostin inhibits differentiation into fibroblast lineages, collagen secretion and matrix compaction resulting in elongated and myxomatous valves. With respect to mechanism(s), PN promotes matrix compaction by directly binding to collagen or indirectly by binding to β integrins and altering cytoskeleton organization that modify matrix compaction. Specifically, to test the latter mechanism, we initiated signaling studies to determine if PN promoted valve compaction and maturation by activating β3(β1) integrin-associated signaling and if such activation included phosphorylation of filamin-A (FLNA), an actin-binding protein expressed in valve interstitial fibroblasts. IP data, western blots and immunostaining indic ated that PN binding to β 3 integrin specifically promoted phosphorylation of FLNA (2152) by activating cdc42 and pak1 kinases and that 2152 phosphorylation site of FLNA occurred near two point mutations (P673Q,G228R) found in patients with degenerative (myxomatous) valve diseases. Silencing periostin inhibited FLNA (2152) phosphorylation which correlated with reduced potential of prevalvular interstitial cells to compact collagen gels. Similarly, we also found that introducing the P673Q and G228R mutations into these cells also inhibited collagen compaction and the phosphorylation of FLNA (2152). Supported by NHLBI, AHA, NCRR and Leducq Mitral Transatlantic Network

8.14 Fibulin-1 regulation of cell cycle progression in developing cardiac valves

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Fibulin-1 (Fbln1) is a component of the extracellular matrix of endocardial cushions and adult cardiac valves, but its function in the process of cardiac valvulogenesis is not known. Analysis of Fbln1 null embryonic hearts revealed that aortic and pulmonary valves are smaller (30%; $p<0.05$) and have fewer cells (25%; $p\leq0.05$) as compared to the wildtype valves. To determine if the reduced size and hypocellularity of Fbln1 null valves was due to apoptosis we performed TUNEL analysis, but found no difference in the level of apoptosis between wildtype and Fbln1 null valves. We next determined whether decreased proliferation contributed to the phenotype. Paradoxically, we observed a 20% increase ($p<0.05$) in Ki67 and a 10% increase ($p<0.01$) in PHH3-positive cells in Fbln1 null aortic and pulmonary valves. However, we also observed increased senescence-associated heterochromatin foci in the nuclei of Fbln1 null valve cells. Additionally, microarray analysis of Fbln1 null E12.5 hearts showed up regulation of an inhibitor of cell cycle progression, p27kip1, and down regulation of Skp1a, a component of ubiquitin ligase complex that degrades p27kip1. The findings suggest that Fbln1 deficiency disrupts cell cycle progression and leads to cellular senescence. In support of this we showed that mouse embryo fibroblasts from Fbln1 nulls stop dividing beyond passage 3, are multinucleated and display arrest in the G2/M phase of mitosis. Taken together, we conclude that Fbln1 is required for proper cell cycle progression in developing valves and perhaps other tissues where it functions to prevent mitotic catastrophe leading to cellular senescence. This work was supported from NIH grant HL095067.

8.15 MTA1-NuRD Chromatin Remodeling Complex is required for Cardiac Valve Development

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Chromatin remodeling is one method of gene regulation that has received increasing attention over the last several years. Our previous work has demonstrated the importance of the transcriptional repressor FOG-2 for the transcriptional regulation of cardiac development; we have shown that FOG-2 functions to repress gene expression through recruitment of the Nucleosome Remodeling and Histone Deacetylase (NuRD) chromatin remodeling complex. This complex is composed of a number of subunits that include Mi2b, Histone Deacetylases 1 & 2, Metastasis Associated Proteins 1-3 (MTA1-3), and the Rb-Associated Proteins. There are at least three distinct NuRD complexes that contain different MTA family members (MTA1-, MTA2-, and MTA3-NuRD) that may have unique roles during development. Here we report the preliminary characterization of a mouse with a targeted disruption in the MTA1 subunit of the NuRD complex. It is our hypothesis that MTA1-NuRD is a distinct NuRD complex that modulates chromatin structure in a non-redundant fashion during heart development. Consistent with this notion, we have observed that MTA1 deficient mice exhibit nearly complete perinatal lethality. Through histological analysis, we have observed that these mice have valvular hyperplasia and atrial septal defects. These defects are accompanied by a decrease in left ventricular fractional shortening, as determined by echocardiography on newborn pups. Taken together, these results demonstrate that the MTA1-NuRD complex is critical for normal cardiac development and provides further evidence for the importance of chromatin remodeling in this process.

8.16 The Ventricular Growth Signal is Generated by Atrio-Ventricular Valve Flow; A Study Using Infants with Congenital Heart Disease As Models.

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Objectives: To determine the signal for ventricular and atrioventricular (AV) valve growth, infants with congenital heart lesions served as models. Our basic hypothesis was that clinical ventricular hypoplasia is developmental, rather than primarily genetic, and catch-up growth can be induced with the correct biomechanical stimulus. The corollary hypothesis was that forward flow across the AV valve (mitral or tricuspid) generates the growth signal. These hypotheses were tested in patients who had procedures to increase AV valve flow to attempt to correct a hypoplastic ventricle. **Methods:** Ventricular volumes (RV, LV) were assessed by biplane echo and indexed to body surface area (m^2) and the deviation from expected volumes calculated as standard error of mean (SEM). Hypoplasia was considered significant when the SEM < -2.0. The groups were

studied before and after (3-6 months) procedures which increased AV flow. **Results:** In unbalanced AV canal defects, the ventricles went from very hypoplastic 9.8 – 19.4 cc/m^2 (-7.1 to -4.2 SEM) to normal range 32 – 45 cc/m^2 (-1.2 to -0.1 SEM). In pulmonary atresia with intact ventricular septum, right ventricular volumes 2.5 ± 1.1 cc/m^2 (-5.1 ± 2.5 SEM) grew to 19.4 ± 4.6 cc/m^2 (-1.5 ± 1.9 SEM). Other possible mechanisms (high wall stress or significant retrograde flow from semilunar valve regurgitation) did not produce catch-up ventricular growth. **Conclusions:** 1) Congenital heart defects can serve as models for basic developmental questions. 2) Increased AV valve flow provides the ventricular growth signal. 3) Operations which increased AV valve flow induced catch-up growth of hypoplastic ventricles.

8.17 MAPK and twist1 mediate scleraxis expression to regulate chondroitin sulfate proteoglycans in developing heart valves.

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In contrast to normal valves, diseased myxomatous mitral valves (MMV) are pathologically thickened and display defects in connective tissue organization, associated with excess chondroitin sulfate proteoglycan (CSPG) deposition and functional prolapse. Despite the clinical significance, the signaling pathways required for regulation of CSPG deposition in developing and mature heart valves are not fully understood. The basic helix-loop-helix (bHLH) transcription factor scleraxis (*scx*) is highly expressed in developing valve structures and null mice display disorganized connective tissue and attenuated CSPG expression. In support, scx gain-of-function in vitro increases CSPG expression, and additional transfection studies of *scx* mutant constructs in C3H10T1/2 cells suggest that the DNA-binding domain is required to promote CSPG expression. To determine the molecular mechanisms of scx-mediated CSPG regulation, we employed an established in vitro chicken valve precursor (VP) cell culture system. As studies have shown that MAPK signaling can influence *scx*, we treated VP cells with adenovirus targeting MEK1/2. Forty-eight-hour treatment with constitutively active MEK1/2 adenovirus (AdV-caMEK1/2) significantly decreased *scx* (4.8-fold) and CSPG expression, while dominant negative MEK1/2 adenovirus (AdV-dnMEK1/2) resulted in an increase in *scx* (9.5-fold) and CSPG expression. As ERK1/2 phosphorylation sites are absent on scx, we hypothesized that MAPK regulation is indirect; and pervious studies suggest bHLH repressor twist1 as a signaling mediator. In our system, AdV-caMEK1/2 treatment dramatically increased twist1 protein levels, and 48-hr AdV-twist1 treatment led to a significant decrease (4-fold) in *scx* expression. Ongoing studies will examine the role of MAPK-twist1-scx signaling pathways in MMV disease in human patients and mouse models.

8.18 Filamin-A Functions as a Rheostat to Control Synthesis of Matrix Proteins During Valve Development and Disease

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Linkage and sequencing studies of patients with myxomatous valvular dystrophy revealed causal mutations in the Filamin-A gene. These studies defined Filamin-A as integral to valve structure and function. However, the mechanisms by which Filamin-A regulates valve cell biology are unknown. Herein we demonstrate that Filamin-A can function as a rheostat to precisely control the amount and accumulation of extracellular matrix proteins during valve development. Our studies define that Filamin-A, through its interactions with the MAPK signal transducer, r-Ras can regulate the phospho-status of pErk1/2. We further define that a normal function of pErk1/2 is to suppress nuclear translocation of pSmad3 by phosphorylating the Smad3 linker region. Filamin-A deficient valves show a near complete loss of r-Ras and pErk1/2 activity and a subsequent increase in Smad3 C-terminal phosphorylation and nuclear accumulation. By neonatal timepoints, a significant increase in nuclear pSmad3 levels is observed concomitant with increased production of fibrillar extracellular matrix proteins. Dysregulated matrix synthesis in the Filamin-A deficient mouse continues throughout postnatal life progressing to a myxomatous valvular dystrophy by 2 months of age. Thus, our studies indicate matrix synthesis during valve development may be tightly regulated through a novel yin-yang interaction between MAPK pathways and

TGFb/Smad3 pathways and are dependent on Filamin-A acting as a rheostat. As Filamin-A point mutations causal to human myxomatous valvular dystrophy occur in the r-Ras binding domain, our studies suggest new mechanisms by which matrix is synthesized during valve development as well as providing insight into mechanisms underlying degenerative cardiac valve diseases.

8.19 Differentiation of heart valve progenitor cells is dependent on communication between periostin-integrin and hyaluronan-induced signaling

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Periostin (PN) is a fasciclin-related protein secreted by prevalvular mesenchyme of the ventricular inlets and outlets and interstitial cells of the ventricular myocardium. We have previously shown that PN promotes cell autonomous, fibrogenic-differentiation and compaction of collagenous matrix into mature valve. Loss of periostin inhibits differentiation into fibroblast lineages, collagen secretion and matrix compaction resulting in enlongated valves with potential for regurgitation. we investigated PN, as a matricellular protein, has potential to signal for valve compaction and maturation by activating β3/(β1) integrin-associated pathways and determined if such activation has cross-talk with hyaluronan signaling. Our recent studies demonstrate that: (1) PN increases hyaluronan secretion via PN/integrin/PI3K signaling in embryonic valve cells, (2) PN-induced signaling communicates with hyaluronan/CD44 interacted signaling, (3) PN interacts with specific integr in β3 downstream PI3K/AKT mediated cell survival to stimulate adhesion/growth and AV cell survival, (3) PN/integrin-β1 interaction activate cytoskeletal associated filamin-A, and (5) periostin and hyaluronan are co-expressed in wild type E16.5 mouse valve sections, whereas hyaluronan expression is suppressed in E16.5 periostin null mouse valve sections. Together, these findings indicate that PN and hyaluronan can regulate cytoskeletal changes required for valve maturation by triggering signaling mechanisms that activate cytoskeletal FLNA. Supported by NHLBI, AHA, NCRR and Leducq Mitral Transatlantic Network.

8.20 Bicuspid Aortic Valve Leaflets Experience Increased Stretch which Activates Inflammatory Gene Expression.

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Aortic valve calcification is the third leading cause of adult heart disease and the most common form of acquired valvular disease in developed countries. The risk factor most closely linked to calcific aortic stenosis is bicuspid aortic valve (BAV). Examination of calcified aortic valve leaflets demonstrates that inflammatory changes are involved. There is no effective treatment other than valve replacement. Additional understanding of the molecular pathogenesis of aortic valve calcification is required to develop effective medical treatments for this disease. To determine if the fused leaflet of BAVs experience more stretch than tricuspid aortic valves (TAVs), we performed *ex vivo* experiments comparing porcine TAV valves to surgically created porcine BAVs. We found that BAVs experience 25% stretch in the radial direction as compared to 5% for TAVs. To determine if increased stretch altered gene expression in human aortic valve interstitial cells (AVICs), we used microarrays to profile the RNA expression profiles between AVICs exposed to static condition or 14% stretch at 1hz for 24h. *INTERLEUKIN1B* (*IL1B)* and *MATRIX METALLOPROTEINASE-1* (*MMP1*) were among the most stretch unregulated genes. These genes are found at higher levels in calcified aortic valves. Additional *INTERLEUKIN* and *MATRIX METALLOPROTEINASE* family members were also unregulated by stretch. The expression changes were confirmed by qPCR. Our data demonstrates that the fused leaflet of BAV experiences increased stretch and that this stretch is sufficient to activate some of the inflammatory genes that are expressed at higher levels in calcified aortic valves.

8.21 4-D Shear Stress Maps of the Developing Heart using Doppler Optical Coherence Tomography

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Accurate imaging and measurement of hemodynamic forces is vital for investigating how physical forces acting on the embryonic heart are transduced and influence developmental pathways. However, making these measurements in the functioning embryonic heart is difficult due to its diminutive size and rapidly changing, pulsatile blood flow. Of particular importance is blood flow-induced shear stress, which influences gene expression by endothelial cells and potentially leads to congenital heart defects through abnormal heart looping, septation, and valvulogenesis. Previous efforts to measure the shear stress on the endocardium have not succeeded in mapping these patterns in 3-D and 4-D. Using 4-D structural and Doppler OCT, we are able to accurately measure the blood flow in the heart tube *in vivo* and to map endocardial shear stress throughout the heart cycle under physiological conditions. These shear stress measurements were compared with immunostaining patterns of KLF2, a shear responsive protein. KLF2 was highly expressed along the inner curvature of the outflow tract which corresponds well with our shear stress measurements. These combined measurements of the beating heart will be valuable in determining the influence of shear force on normal and abnormal development and may be sensitive enough to detect the earliest signs of congenital heart defects. For the first time, we can directly measure the pattern of shear stress on the endocardium of the developing heart tube in 4-D, which will enable precise titration of experimental perturbations and accurate correlation of shear with expression of molecules critical to heart development. (RO1HL083048, R01HL095717, T32EB007509, C06RR12463-01)

8.22 The origin and the role of hematopoietic-derived cells in embryonic heart valve development

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Our group has previously described the presence of $CD45^+$ cells in the developing cardiac valves and their contribution to homeostasis and pathological remodeling of the post-natal valves. We have shown that $CD45^+$ cells in the post-natal valves originate from the hematopoietic compartment of the bone marrow. However, the source of these cells in the embryonic valves is unclear. To determine whether CD45 might be expressed by endocardially-derived cells that populate the endocardial cushions, or is expressed only on valve cells of extra-cardiac origin, chick cushions from stages prior (ED3) and after (ED5) $CD45^+$ cells populate them in vivo were explanted onto collagen gels and cultured for up to three days. Using this approach, we never detected $CD45^+$ cells in the ED3 explants, suggesting that CD45 is not expressed on endocardially-derived cushion cells. This finding is supported by our quail-chick chimera studies, where the quail aorta was transplanted into chicken embryos. $QH1^+$ cells were detected interspersed between host $CD45^+$ cells in the cushion mesenchyme, highlighting the ability of embryonic hematopoietic cells to migrate into the developing heart valves. Next, to begin to evaluate whether $CD45^+$ cells might contribute to valve development, we used CCN1-null mice in order to prevent the invasion of $CD45^+$ cells into the developing cushions. Along with the already described malformations in CCN1-null hearts, we observed significantly reduced $CD45^+$ cell numbers in the cushions. Collectively, our findings suggest that hematopoietic-derived ($CD45^+$) cells migrate into the developing valves and perturbation of their invasion may contribute to the cardiac malformations.

8.23 The role of BMP in differentiation and lineage restriction of AV endocardial cushion cells

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Formation and distal outgrowth of the endocardial cushion mesenchyme in the atrioventricular (AV) canal is the first and essential morphogenetic event in cardiac valvuloseptal morphogenesis. AV endocardial cushion mesenchymal cells (ECMCs) likely have the potential to abnormally differentiate into other mesodermal cell lineages. BMPs are multifaceted regulators that regulate cell differentiation in a cell-type dependent fashion. Our current research tested the hypothesis that BMP-2 signaling accomplishes two-fold functions of i) promoting ECMC differentiation into pre-valvular fibroblasts and ii) inhibiting differentiation of alternative mesodermal cell

fates via intersection with other cell fate determinants like Notch pathway. This hypothesis is supported by our data that BMP-2 ligand and BMP type I receptors, Alk3 and Alk2, as well as notch receptors Notch ½ are expressed in ECMCs during AV cushion maturation and that BMP-2 induces expression of Notch ½ and N otch pathway intermediates in the ECMC aggregate culture. BMP-2 induces the valvulogenic phenotype including extracellular matrix (ECM) protein expression and cellular migration and also inhibits expression of alternative mesodermal lineage markers in chick ECMC cultures. To complement this in vitro finding, we also tested the effect of gain and loss of BMP signaling in AV cushion mesenchyme in vivo using an endocardium-specific cre-driver line. Alk3 endocardial conditional knock-out mice die by embryonic day 12.5 and have defects in endocardial cushion development and ECM protein expression in ECMCs, whereas endocardial/endocardial cushion specific constitutive up-regulation of Alk3 results in enlarged endocardial cushions with up-regulated pSmad 1/5/8 expression and abundant ECM protein expression in ECMCs.

8.24 Glycerophosphocholines and Sphingolipids are Compartmentalized within Ovine and Human Aortic Valve Substructures and are Differentially Regulated during Ovine Transitional Circulation

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Introduction: Congenital and acquired aortic valve stenosis (AVS) affects over 10 million people with cellular mechanisms mostly unknown. Degeneration of the valve structure involves atherosclerotic-like processes with lipid deregulation and calcification triggered by activated valvular interstitial cells (VIC). Since disease-activated VIC are proposed to recapitulate embryonic programs, we tested the hypothesis that lipids are regulated within the developing aortic valve (AV). **Methods:** Ovine AV at prenatal day 5 and adult (each n=3), de-identified human AV leaflets from pediatric patients with severe AVS (n=2) were examined for lipid distribution using matrix-assisted laser desorption ionization imaging mass spectrometry. Lipid alteration in AV substructure was modeled by receiver operating characteristic curve ≥0.8 with spectral sets >100 per each valve region. Valve extracellular matrix (ECM) was evaluated by Movat's pentachrome and Verhoeff-Van Geison stain. Co-expression of endothelial differentiation gene 1 (EDG1) and α smooth muscle actin, a marker of aVIC, was investigated by immunofluorescence. **Results/Conclusions:** Glycerophosphocholines and sphingolipids were spatially restricted and regulated within human and ovine AV substructures. Expression patterns followed ECM stratification. Lipid signatures from ovine fetal and adult substructures showed alteration in 18 lipid species. Principle component analysis showed distinct clustering between ovine timepoints. EDG1, the receptor for sphingosine-1-phosphate, was expressed by VIC at ovine fetal and adult timepoints with decreased expression in the aortic wall. Lipids followed aberrant ECM in human pediatric AVS. This study defines a novel role for lipids in valve development and provides a new approach to investigating lipid involvement in AV disease.

8.25 Fibulin-1 regulation of cell cycle progression in developing cardiac valves

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Fibulin-1 (Fbln1) is a component of the extracellular matrix of endocardial cushions and adult cardiac valves, but its function in the process of cardiac valvulogenesis is not known. Analysis of Fbln1 null embryonic hearts revealed that aortic and pulmonary valves are smaller (30%; $p<0.05$) and have fewer cells (25%; $p\leq0.05$) as compared to the wildtype valves. To determine if the reduced size and hypocellularity of Fbln1 null valves was due to apoptosis we performed TUNEL analysis, but found no difference in the level of apoptosis between wildtype and Fbln1 null valves. We next determined whether decreased proliferation contributed to the phenotype. Paradoxically, we observed a 20% increase ($p<0.05$) in Ki67 and a 10% increase ($p<0.01$) in PHH3-positive cells in Fbln1 null aortic and pulmonary valves. However, we also observed increased senescence-associated heterochromatin foci in the nuclei of Fbln1 null valve cells. Additionally, microarray analysis of Fbln1 null E12.5 hearts showed up regulation of an inhibitor of cell cycle progression, p27kip1, and down regulation of Skp1a, a component of ubiquitin ligase complex that degrades p27kip1. The findings suggest that Fbln1 deficiency disrupts cell cycle progression and leads to cellular senescence. In support of this we showed that mouse embryo fibroblasts from Fbln1 nulls stop dividing beyond passage 3, are multinucleated and display arrest in the G2/M phase of mitosis. Taken together, we conclude that Fbln1 is required for proper cell cycle

progression in developing valves and perhaps other tissues where it functions to prevent mitotic catastrophe leading to cellular senescence. This work was supported from NIH grant HL095067.

8.26 Direct Role of Hyperphosphatemia in Vascular and Heart Valve Calcification

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The incidence of vascular and aortic valve calcification is significantly increased in patients with chronic kidney disease leading to mortality related to cardiovascular events. This pathological calcification has been associated with hyperphosphatemia and elevated serum levels of FGF23, however the direct effects of these circulating factors on valve calcification has not been examined. To address this, pig aortic valve interstitial cells (pAVICS) and murine aortic valve (mAoV) explants were treated with 1-3mM phosphate-supplemented media or 25-100ng/mL FGF23 for 3-8 days. Following treatment with phosphate, but not FGF23, pAVICs and mAoV explants cells demonstrate a significant increase in Von Kossa reactivity (213 and 109 fold increase, $p<0.05$). As expected, the mouse vascular smooth muscle (MOVAS) cell line similarly demonstrated matrix mineralization, associated with increased expression of Runx2, Spp1 and BMP2 in a dose and time-dependent manner. To determine if phosphate-mediated calcification in heart valves requires phosphate transporters, pAVICs and mAoV explants were treated with phosphate in the presence of the phosphate transporter inhibitor, phosphonoformic acid (PFA). In both pAVICs and mAoV, PFA treatment (0.1M) significantly attenuated (67 and 88 fold decrease, $p<0.05$) phosphate-induced Von Kossa reactivity and was comparable with levels observed in controls (3 and 1 fold increase, not significant). Ongoing studies are focused on examining the molecular mechanisms that underlie phosphate-induced calcification of heart valves. Overall, these studies suggest that phosphate, but not FGF23 can directly promote calcification, thereby providing insights into mechanisms of pathogenesis and identifying targets of therapeutic intervention for vascular and valve calcification.

8.27 Do the valve endocardial progenitors originate from a single zebrafish blastula HPRG1$^+$ cell?

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The arguments regarding the origin of the endocardial progenitors remain unresolved (Harris and Black, 2010). Here, we have identified a gene, tentatively named *HPRG1* (heart progenitor regulation gene 1), through a large-scale screen of *Drosophila* mutants. The gene is expressed in heart valves in zebrafish and its expression pattern is conserved in mice. Knockdown of the gene resulted in a valve defect, suggesting it is involved in endocardial valve development. It is known that *Isl1* or *GATA4* positive cells are capable of differentiating into two cell types, endocardial and cardial progenitors, and *NKx2.5* is the direct activator of endocardial master regulator *Etv2*. Our results indicated that HPRG1 is expressed in a novel type of mesodermal progenitor cells that are co-expressed with each master regulators and HPRG1 activates the expressions of GATA4 and NKx2.5 and inhibits the expression of Isl1. It is specially interesting that HPRG1 determines the fate of a single cell of the 128-cells at Zebrafish blastula stage, suggesting that it is a fate-determining gene. Thus, the HPRG1 positive blastula cells provide an appropriate experimental system for exploring the specification mechanism of the endocardial progenitors. A mechanism for heart valve progenitor specification beginning with *HPRG1* through *GATA4*, *Isl1* and *NKx2.5* is under investigation.

8.28 Hemodynamics-dependent valvulogenesis of zebrafish heart mediated by miR-21

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Recent researches have revealed that atrioventricular valves (AVV) formation requires mechanical forces such as a beating heart and blood flow. Physical disturbance of zebrafish heart beat results in defective AVV formation, similar to that observed in congenital heart diseases. The aim of our current research is to figure out the unknown mediator between mechanical forces and genetic programs during valvulogenesis. We hypothesized that microRNAs (miRs) are one of the possible candidates for two reasons. First, some miRs are involved in cardiac defects and dysfunctions. Secondly, pressure overload on hearts induces some miRs transcription, implying their mechano-dependent expression. Hence, we searched miRs expressed in zebrafish hearts and found that miR-21 is specifically detectable in the endocardium of developing AVV.

This valve specific miR-21 expression was downregulated when the heart beat was blocked by BDM (myosin specific ATPase inhibitor), while it was promptly restored by re-initiating the heart beat. These results suggest a hemodynamic-dependent expression of miR-21. Knock down of miR-21 by antisense morpholino oligo induced hypoplastic AVV. Endocardial cells in the valve forming region failed to proliferate in miR-21 morphants, indicating that miR-21 plays a cell growth-related role in response to the heart beat. All in all, miR-21 expression triggered by mechanical stresses contributes to zebrafish AVV formation. We also show updated data on the function of miR-21 promoter/enhancer in responding to mechanical stresses.

8.29 W-Loop Of Alpha-Cardiac Actin Is Critical For Heart Function And Endocardial Cushion Morphogenesis In Zebrafish

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Mutations in cardiac actin (ACTC) have been associated with different types of cardiac abnormalities in humans including dilated cardiomyopathy and septal defects. However, it is still poorly understood how altered ACTC structure affects cardiovascular physiology and results in the development of distinct congenital disorders. A cardiovascular-specific zebrafish mutant (s434) was identified that displays blood regurgitation in a dilated heart between the atrium and ventricle and lacks endocardial cushion formation. We identified the mutation as a single nucleotide change in *Alpha-Cardiac Actin (actc1a)*, resulting in a Y169S amino acid substitution. This mutation affects the W-loop of actin, which has been implicated in nucleotide sensing. Our results demonstrate that s434 mutants show loss of polymerized cardiac actin. An analogous mutation in yeast results in rapid depolymerization of F-actin into fragments that are not able to reanneal. This polymerization defect can be partially rescued both in yeast and zebrafish embryos by phalloidin treatment which stabilizes F-actin. Physiological analyses reveal that *actc1a* mutant embryos show defects in cardiac contractility and altered blood flow within the heart tube. As a result, molecular markers for endocardial cushion formation including *notch1b*, *nfatc1*, *versican* and *klf2a* are downregulated or mislocalized in s434 mutants, leading to the absence of EC development. Our study underscores the importance of the W-loop for actin functionality and will help us to understand structural and physiological consequences of certain ACTC mutations in human congenital disorders.

8.30 Olfactomedin-1 activity identifies a cell invasion checkpoint during epithelial-mesenchymal transition in the embryonic heart.

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Endothelia in the atrioventricular (AV) canal of the developing heart undergo an epithelial mesenchymal transition (EMT) and invade the underlying extracellular matrix to begin heart valve formation. Using an *in vitro* invasion assay, an extracellular matrix protein found in the heart, Olfactomedin-1 (OLFM1), increases mesenchymal cell numbers and anti-OLFM1 antibody inhibits normal mesenchymal cell formation. OLFM1 does not alter cell proliferation, migration or apoptosis. Dispersion, but lack of invasion in the presence of inhibiting antibody, identifies a role for OLFM1 in cell invasion during EMT. To explore OLFM1 activity during EMT, representative EMT markers were examined. Effects of OLFM1 and anti-OLFM1 on cell-cell adhesion molecules and the transcription factors, Snail-1, Snail-2, Twist1, and Sox-9 are inconsistent with initiation of EMT. Transcription factors, Zeb1 and Zeb2, secreted proteases and several mesenchymal cell markers are regulated by OLFM1 and anti-OLFM1 consistent with regulation of invasion. We conclude that OLFM1 is present and necessary for EMT in the developing heart. Its role in cell invasion and mesenchymal cell gene expression argues for an invasion checkpoint in EMT where additional signals are required to initiate cell invasion into the three-dimensional matrix. Incubation of several cancer cell lines with exogenous OLFM1 suggests a conserved role in other EMTs.

8.31 Twist1 Homodimer and Heterodimer Function in Heart Valve Development

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Heart valves develop from extracellular matrix-rich endocardial cushions (ECCs) populated by proliferative, migratory, and undifferentiated mesenchymal cells. The basic helix-loop-helix transcription factor, Twist1 is highly expressed in ECCs, but not remodeling valves, and is re-expressed during pediatric and adult heart valve disease. Twist1 binds to E-box consensus sequences (CANNTG) as a homodimer or a heterodimer with other bHLH proteins, including E12/E47. Twist1 dimers have differential functions and distinct gene targets in murine cranial suture and limb development and also in Drosophila mesoderm cell lineage determination. We hypothesize that Twist1 homo- and heterodimers differentially regulate gene expression and developmental functions during heart valve development. In previous studies, we identified *Tbx20, Cdh11*, *Col2a1*, *Sema3C*, *Rab39b*, and *Gadd45a* as direct transcriptional targets of Twist1 during heart valve development. Differential binding of endogenous Twist1 and E12/E47 to known Twist1 target gene enhancers was observed by chromatin immunoprecipitation (ChIP) assays. Differential functions of Twist1 homo- and heterodimers were examined using tethered Twist1-Twist1 (TT) and Twist1-E12 (TE) dimer overexpression in cultured cells and transgenic mice. In MC3T3-E1 preosteoblast cells, expression of Twist1 target genes is differentially affected by transfection of TT or TE tethered dimers. In preliminary studies, mice with conditional loss of Twist1 or expression of Twist1 tethered homo- and heterodimers have abnormal ECCs and valve morphogenetic defects. Together these studies provide initial evidence for differential functions of Twist1 homo- and heterodimers in target gene expression and heart valve development.

8.32 The roles of Smad8 in calcific aortic valves

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Among heart diseases, those affecting the cardiac valves are the most common. Within this group, aortic valve calcification is especially prevalent as the population ages. The incident of aortic valve calcification increases with age and affects up to 20% of the population over the age of 80. Although such a prevalent medical problem, the genetic bases and molecular mechanism underlying how valve diseases develop, in particular how valve calcification develops, is poorly understood. Among the molecules involved in cardiac valve development and pathogenesis, Bone Morphogenetic Protein (BMP) signaling has been shown to be essential in aortic valve development and their misregulation also leads to calcification of aortic valves. We have found that systemic knockout of Smad8, one of the intercellular components of BMP signal transduction pathway, develops valve defects including disorganization of the extracellular matrix layers of valves and their calcification in adult mouse. Smad8 null mice do not develop other major structural cardiac defects making them an ideal model to study the roles of BMP signaling in cardiac valve disease. We have found that in Smad8 null mice, other members of the Smad family, Smad1, 2, and 5, are phosphorylated leading to nuclear localization. Our data suggests that a negative feedback loop within the Smad pathways regulates development of cardiac valves and their misregulation leads to diseases of valves in adults.

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