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Weinstein Cardiovascular Development Conference

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Welcome to Indy!

On behalf of the local organizing committee, we would like to welcome you to the 2007 Weinstein Cardiovascular Development Conference. It is our privilege and honor to host this year's conference. Since it was first convened in 1994, the Weinstein conference has evolved into the preeminent conference for cardiac development worldwide. This annual meeting provides a unique forum in which to share the latest discoveries, novel concepts, and cutting edge technologies for understanding formation of the heart and vasculature. We look forward to another successful gathering in 2007. Two distinguished scientists, Drs. Oliver Smithies and Margaret Kirby, are our keynote speakers. Dr. Smithies has a long and distinguished career in molecular genetics, and is largely credited with the co-development of gene targeting techniques. Dr. Kirby is known world wide for her work on the role of neural crest cells in the genesis of congenital heart defects. This year's keynote speakers were selected to continue the emphasis that the Weinstein Conference places on young scientists; Dr. Smithies and Dr. Kirby are both exemplary role models for young investigators.

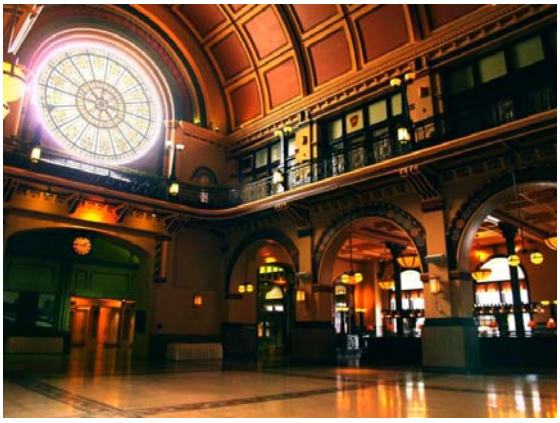
The organizing committee received over 200 abstracts, from which 42 were selected for platform presentation. The platform sessions represent a broad spectrum of topics relevant to cardiovascular development. The Weinstein Conference traditionally tries to encourage the participation of young investigators. To attain this goal, platform presentations have been awarded predominantly to postdoctoral fellows and young investigators below the rank of Associate Professor. We have also included four "Workshops" in the program to foster discussion and debate on topical areas of cardiac development. As always, the poster sessions will constitute the main focal point of the conference, and space will be provided so that the posters can be displayed for the entire meeting. We hope that you will have a memorable conference and an inspiring experience in Indianapolis.

2007 Local Organizing Committee:

Simon Conway	Loren Field	Tony Firulli	Mark Payne
Michael Rubart	Weinian Shou	Lei Wei	

Ad hoc members:

Michiko Watanabe Katherine Yutzey



Venue: The Crowne Plaza and Conference Center at Historic Union Station

SCHEDULE AT A GLANCE

Thursday, May 10, 2007

10:00 am – 4:00 pm	Registration	Grand Hall
1:45 pm	Opening remarks	Illinois Street Ballroom
2:00 pm – 3:05 pm	Platform Session I	Illinois Street Ballroom
3:05 pm – 3:20 pm	Break	
3:20 pm – 4:25 pm	Platform Session II	Illinois Street Ballroom
4:25 pm – 4:45 pm	Break	
4:45pm – 6:00 pm	Keynote Presentation by Dr. Oliver Smithies	Illinois Street Ballroom
6:00 pm – 8:00 pm	Buffet Dinner & Poster Session 1 (even) (Open bar 6:00 pm – 10:00pm)	Grand Hall Ballroom
8:00 pm – 9:00 pm	Concurrent Workshops	Edison Rooms

Friday, May 11, 2007

7:30 am – 9:00 am	Breakfast	Grand Hall Ballroom
9:00 am – 10:20 am	Platform Session III	Illinois Street Ballroom
10:20 am – 10:35 am	Break	
10:35 am – 12:00 pm	Platform Session IV	Illinois Street Ballroom
12:00 pm – 1:15 pm	Lunch	Grand Hall Ballroom
1:15 pm – 2:15 pm	Concurrent Workshops	Edison Rooms
2:15 pm – 2:40 pm	Break	
2:40 pm – 4:00 pm	Platform Session V	Illinois Street Ballroom
4:00 pm – 4:30 pm	Break	
4:30 pm – 5:45 pm	Keynote Presentation by Dr. Margaret Kirby	Illinois Street Ballroom
6:00 pm – 8:00 pm	Buffet Dinner & Poster Session 2 (odd) (Open bar 6:00 pm – 10:00pm)	Grand Hall Ballroom

Saturday, May 12, 2007

7:30 am – 9:00 am	Breakfast	Grand Hall Ballroom
9:00 am – 10:20 am	Platform Session VI	Illinois Street Ballroom
10:20 am – 10:35 am	Break	
10:35 am – 12:10 pm	Platform Session VII	
12:15 pm – 1:30 pm	Lunch	Grand Hall Ballroom
1:30 pm – 2:30 pm	Business Meeting NHLBI Workshop	Illinois Street Ballroom Edison Room
2:30 pm – 2:45 pm	Break	
2:45 pm – 4:05 pm	Platform Session VIII	Illinois Street Ballroom
4:05 pm – 4:30 pm	Break	
4:30 pm – 5:35 pm	Platform Session IX	Illinois Street Ballroom
6:00 pm – 10:00 pm	Banquet	Grand Hall Ballroom

FULL CONFERENCE SCHEDULE

(Note: presenting author is underlined)

Thursday, May 10, 2007

10:00 am - 4:00 pm **Registration** **Grand Hall**

1:45 pm **Opening remarks** **Illinois Street Ballroom**

2:00 pm – 3:05 pm **Platform Session I** **Illinois Street Ballroom**

Cardiomyocyte cell cycle regulation

Chairs: Loren Field, Indiana University and Youngsook Lee, University of Wisconsin Medical School

2:00 – 2:05 **Chair's Introduction**

2:05 – 2:20 **Cardiomyocyte and Epicardial Cell Addition during Cardiac Homeostasis in Adult Zebrafish**

Wills, Airon A; Holdway, Jennifer; Major, Robert J; Poss, Kenneth D

Duke University Medical Center, Dept. of Cell Biology

2:20 – 2:35 **FOXO transcription factors in the regulation of cardiac myocyte proliferation and myocardial growth during development**

Evans-Anderson, Heather J; Alfieri, Christina M; Yutzey, Katherine E

Division of Molecular Cardiovascular Biology, Cincinnati Children's Hospital Medical Center

2:35 – 2:50 **3D Visualization of proliferation in cardiac and extracardiac mesoderm**

van den Berg, G; Soufan, AT; de Boer, PAJ; van den Hoff, MJB; Moorman, AFM

Heart Failure Research Centre, Amsterdam, The Netherlands

2:50 – 3:05 **Canonical Wnt Signaling is Required for Mammalian Cardiogenesis by Regulating Cardiac Progenitors**

Kwon, Chulan; Arnold, Joshua; Taketo, Makoto; Srivastava, Deepak

Gladstone Institute, UCSF

3:05 pm – 3:20 pm **Break**

3:20 pm – 4:25 pm **Platform Session II** **Illinois Street Ballroom**

Myofibrogenesis

Chairs: Lei Wei, Indiana University and Jeffrey Robbins, Cincinnati Children's Hospital Medical Center

3:20 – 3:25 **Chair's Introduction**

3:25 – 3:40 **MEF2A controls a costameric network of genes in cardiac muscle**

Naya, Frank J; Brand, Ondra M; Reynolds, Joseph G; McCalmon, Sarah A

Department of Biology, Program in Cell and Molecular Biology, Boston University

3:40 – 3:55 **Investigating the role of Prox1 in the developing heart**
Risebro, Catherine A (1); Searles, Richelle G (1); Melville, Athalie AD (1); Ehler, Elisabeth (2); Harvey, Natasha L (3); Dillard, Miriam (4); Moses, Kelvin (5); Schwartz, Robert J (5); Chien, Kenneth R (6); Oliver, Guillermo (4); Riley, Paul R (1)
(1) UCL Institute of Child Health, London, England (2) The Randall Centre of Cell and Molecular Biophysics, Guy's Campus, Kings College, London, England (3) The Hanson Institute, Adelaide, Australia (4) St. Jude Children's Research Hospital, Memphis, USA (5) Texas A&M University Health Science Center, Houston, USA (6) MGH Cardiovascular Research Center, Boston, USA

3:55 – 4:10 **High resolution in vivo imaging of the cross-sectional deformations of contracting embryonic heart loops using optical coherence tomography (OCT).**
Manner, Jorg (1); Thrane, Lars (2); Norozi, Kambiz (3); Yelbuz, Mesud T (3)
(1) Department of Anatomy and Embryology, Georg-August-University of Göttingen, Germany (2) Optics & Plasma Research Department, Risø, Denmark (3) Department of Pediatric Cardiology and Intensive Care Medicine, MHH Hannover, Germany

4:10 – 4:25 **Mouse mutants with defects in cardiovascular development isolated from balancer chromosome mutagenesis screens**
Mitchell, Karen (1); Risley, Michael D (1); Green, David (1); Justice, Monica J (2); Hentges, Kathryn E (1)
(1) Faculty of Life Sciences, The University of Manchester, Manchester, England M13 9PT, (2) Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas, USA

4:25 pm – 4:45 pm **Break**

4:45 pm – 6:00 pm **Keynote Presentation 1** **Illinois Street Ballroom**
Dr. Oliver Smithies: "ES Cells: back to the future – the history and promise of embryonic stem cells"

6:00 pm – 8:00 pm **Buffet Dinner and** **Grand Hall Ballroom**
Poster Session 1 (even-numbered posters are manned)

8:00 pm – 9:00 pm **Concurrent Workshops** **Edison Rooms**
Single vs. multiple developmental heart fields
Moderators: Anthony Firulli, Indiana University and Margaret Kirby, Duke University

8:00 – 8:07 *Dr. Silvia Evans, University of California San Diego*
"Cardiac progenitor populations"

8:07 – 8:14 *Dr. Brian Black, University of California San Francisco*
"The right ventricle, outflow tract, and interventricular septum are derived from the second heart field"

8:14 – 8:21 *Dr. Sigolène Meilhac, Institut Pasteur*
"Clonal segregation between the first and second myocardial lineages"

8:21 – 8:28 *Dr. Vincent Christoffels, University of Amsterdam*
"The caudal and posterior second heart field, and the origin of the left ventricle in chicken and mouse"

8:28 – 9:00 *Panel Discussion*

Cardiomyogenic stem cells during development

Moderators: Loren Field, Indiana University and Paul Riley, University College London Institute of Child Health

8:00 – 8:12 *Dr. Steven Kattman, McEwen Centre for Regenerative Mecicine, Toronto*
"ES cell derived cardiovascular progenitor cells"

8:12 – 8:24 *Dr. Nicola Smart, UCL Institute of Child Health, London*
"Epicardial derived vascular progenitors"

8:24 – 8:36 *Dr. Kaomei Guan, Georg-August-University of Goettingen*
"Spermatogonial-derived ES-like cells"

8:36 – 9:00 *Panel Discussion*

6:00 pm – 10:00 pm **Open Bar** **Grand Hall Ballroom**

Friday, May 11, 2007

7:30 am – 9:00 am **Breakfast** **Grand Hall Ballroom**

9:00 am – 10:20 am **Platform Session III** **Illinois Street Ballroom**

Transcription Factors in Cardiogenesis

Chairs: Anthony Firulli, Indiana University and Brian Black, University of California San Francisco

9:00 – 9:05 **Chair's Introduction**

9:05 – 9:20 **Histone deacetylase 1(HDAC1) is Essential for Cardiac Development in Zebrafish**
Zchut, Sigalit (1); Warren, Kerri S (2); Creton, Robbert (3); Kochilas, Lazaros (1)
(1) Pediatric Cardiology, Department of Pediatrics, Rhode Island Hospital Providence, RI (2) Department of Biology, Roger Williams University, Bristol, RI (3) Department of molecular Biology, Cell Biology and Biochemistry, Brown University, Providence, RI

9:20 – 9:35 **In search for partners of Tbx2 and Tbx3.**
Boogerd, Kees J; Wong, LY Elaine; Klarenbeek, M; Christoffels, Vincent M; Moorman, Antoon FM; Barnett, Phil
Heart Failure Research Centre, Academic Medical Centre, Amsterdam, The Netherlands

9:35 – 9:50 **Cooperative function of the transcription factors Nkx2.5 and Mef2c during heart development**
Vincentz, Joshua W; Firulli, Beth A; Firulli, Anthony B
Department of Pediatrics, Wells Center for Pediatric Research, James Whitcomb Riley Hospital for Children, Indiana University School of Medicine Cancer Research Institute

9:50 – 10:05 **Nkx Genes Regulate Heart Tube Extension in Zebrafish**
Targoff, KL; Schell, T; Yelon, D
Developmental Genetics Program and Department of Cell Biology, Skirball Institute of Biomolecular Medicine, New York University School of Medicine, New York, NY

10:05 – 10:20 **DPF3 – a new key transcription factor – bridging chromatin remodeling and cardiac muscle specification**
Lange, Martin (1); Just, Steffen (2); Kaynak, Bogac (1); Dunkel, Ilona (1); Fischer, Jenny J (1); Toenjes, Martje (1); Krueger, Tammo (1); Toedling, Joern (3); Mebus, Siegrun (4); Grimm, Christina (1); Rottbauer, Wolfgang (2); Sperling, Silke (1)
(1) Max Planck Institute for Molecular Genetics, Berlin, Germany; (2) Ruprecht-Karls-University Heidelberg, Germany; (3) European Bioinformatics Institute, European Molecular Biology Laboratory, Cambridge, UK; (4) German Heart Center Berlin, Germany

10:20 am – 10:35 am **Break**

10:35 am – 12:00 pm **Platform Session IV** **Illinois Street Ballroom**
Vascular Development
Chairs: Michiko Watanabe, Case Western Reserve University and Pat Mastin, NIEHS, National Institutes of Health

10:35 – 10:40 **Chair's Introduction**

10:40 – 10:55 **Connexin43 in the Epicardium is Required for Normal Coronary Development**
Zhao, Xiao-Qing; Rhee, David Y; Lo, Cecilia W
Laboratory of Developmental Biology, National Heart, Lung and Blood Institute, National Institutes of Health

10:55 – 11:10 **Endothelial-Specific Ablation of Serum Response Factor Results in Vascular Instability and Embryonic Lethality**
Holtz, Mary L; Misra, Ravi P
Department of Biochemistry, Medical College of Wisconsin, Milwaukee, Wisconsin

11:10 – 11:25 **VEGF-A164 regulates coronary endothelial proliferation, but not tubulogenesis**
Goodwin, Richard L; Nesbitt, Tresa; Yost, Michael J; Potts, Jay D
Department of Cell and Developmental Biology and Anatomy University of South Carolina School of Medicine

11:25 – 11:40 **Canonical Wnt Signaling in Endothelial Cells is Essential for Central Nervous System Vascularization and Blood-Brain Barrier Development**
Stenman, Jan M (1); Carroll, Thomas (2); Rajagopal, Jayaraj (1); McMahon, Andrew P (1)
(1) Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA (2) Department of Internal Medicine (Nephrology) and Molecular Biology, University of Texas Southwestern Medical Center, Dallas, TX

11:40 – 11:55 **Effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on hypoxia-inducible factor-1 alpha during chick cardiogenesis**
Wikenheiser, Jamie (1); Walker, Mary K (2); Watanabe, Michiko (1)
(1) Department of Pediatrics, Case Western Reserve University, Cleveland, OH (2) College of Pharmacy, University of New Mexico Health Sciences Center, Albuquerque, NM.

11:55 – 12:00 **NIH Presentation,** *Pat Mastin, NIEHS*

12:00 pm – 1:15 pm **Lunch** **Grand Hall Ballroom**

1:15 pm – 2:15 pm **Concurrent Workshops** **Edison Rooms**

Cardiac Neural Crest

Moderators: Simon Conway, Indiana University and Anne Moon, University of Utah

1:15 – 1:27 *Dr. Jon Epstein, University of Pennsylvania*
"Cardiac neural crest and interactions with endothelium"

1:27 – 1:39 *Dr. Henry Sucov, University of Southern California*
"Mouse models of PTA that implicate a defect specifically in AP septation"

1:39 – 1:51 *Dr. Rob Gourdie, Medical University of South Carolina*
"Cardiac neural crest ablation inhibits compaction and Electrical function of conduction system bundles"

1:51 – 2:15 *Panel Discussion*

Imaging Cardiovascular Development and Physiology

Moderators: Michael Rubart, Indiana University and Igor R. Efimov, Washington University

1:15 – 1:30 *Dr. Igor R. Efimov, Washington University, St. Louis, MO*
"Biophotonic Imaging of Embryonic Heart"

1:30 – 1:45 *Dr. N. Yvonne Tallini, Cornell University, Ithaca, NY*
"Embryonic Murine Heart using a Genetically Encoded Calcium Inhibitor"

1:45 – 2:00 *Dr. Mary E. Dickinson, Baylor College of Medicine, Houston, TX*
"Imaging fluid motions and heart function in vertebrate embryos"

2:00 – 2:15 *Panel Discussion*

2:15 pm – 2:40 pm **Break**

2:40 pm – 4:00 pm **Platform Session V** **Illinois Street Ballroom**

Cardiac Conduction System Development

Chairs: Michael Rubart, Indiana University and Luis Polo-Parada, University of Missouri, Columbia

2:40 – 2:45 **Chair's Introduction**

2:45 – 3:00 **Molecular and physiological mechanisms underlying embryonic cardiac rhythmicity in zebrafish**
Huang, J (1); Langenbacher, A (1); Goldhaber, J (2); Kown, O (3); Chen, J-N (1)
(1) Department of Molecular, Cell and Developmental Biology, (2) Department of Physiology, and (3) Department of Chemistry and Biochemistry, University of California, Los Angeles

3:00 – 3:15 **Tbx3 acts as a genetic switch for heart pacemaker formation**
Hoogaars, Willem MH (1); Brons, Janynke F (1); Engel, Angela (2); Verkerk, Arie O (2); de Lange, Frederik J (1); Bakker, Martijn L (1); Clout, Danielle E (1); Wakker, Vincent (1); Ravesloot, Jan Hindrik (2); Verheijck, E. Etienne (2); Moorman, Antoon FM (1); Christoffels, Vincent M (1)
(1) Department of Anatomy & Embryology, (2) Department of Physiology, Heart Failure Research Center, Academic Medical Center, University of Amsterdam, Meibergdreef 15, 1105 AZ, The Netherlands.

3:15 – 3:30 **Genetic analysis of Popdc1 and Popdc2 function in mouse and zebrafish heart**
Froese, Alexander (1); Schlüter, Jan (1); Waldeyer, Christoph (2); Kirchmaier, Bettina (1); Breher, Stephanie (1); Liebig, Sonja K (2); Laakmann, Sandra (2); Kirchhof, Paulus (2); Neumann, Joachim (3); Winkler, Christoph (4); Vauti, Franz (5); Arnold, Hans-Henning (5); Fabritz, Larissa (2); Brand, Thomas (1)
(1) Cell- & Developmental Biology, University of Würzburg, Germany, (2) Department of Cardiology and Angiology, University Hospital Münster, Germany, (3) Institute of Pharmacology, University of Halle, Germany, (4) Chemistry I, Biocenter, University of Würzburg, Germany, (5) Cell- & Molecular Biology, TU Braunschweig, Germany

3:30 – 3:45 **Clonal analysis of the origin of the mammalian ventricular conduction system**
Miquerol, Lucile; Moreno, Natividad; Meilhac, Sigolene; Buckingham, Margaret; Franco, Diego; Kelly, Robert G
Inserm Avenir group, Developmental Biology Institute of Marseilles – Luminy, CNRS UMR6216, Marseille, France

3:45 – 4:00 **Deletion of the cardiac L-type calcium channel (CaV1.2) causes embryonic death**
Porter, Jr., George A; Sharma, Ashwani
Yale University School of Medicine

4:00 pm – 4:30 pm **Break**

4:30 pm – 5:45 pm **Keynote Presentation 2** **Illinois Street Ballroom**
Dr. Margaret Kirby: "How many heart fields does it take to make a heart?"

6:00 pm – 8:00 pm **Buffet Dinner and Poster Session 2** (odd-numbered posters are manned) **Grand Hall Ballroom**

6:00 pm – 10:00 pm **Open Bar** **Grand Hall Ballroom**

Saturday, May 12, 2007

7:30 am – 9:00 am **Breakfast** **Grand Hall Ballroom**

9:00 am – 10:20 am **Platform Session VI** **Illinois Street Ballroom**
Signaling Pathways in Cardiogenesis
Chairs: Weinian Shou, Indiana University and Jim Martin, Texas A & M Health Science Center Institute of Biosciences and Technology

9:00 – 9:05 **Chair's Introduction**

9:05 – 9:20 **Endocardial Brg1 Represses ADAMTS1 to Maintain the Microenvironment for Myocardial Morphogenesis**
Stankunas, Kryn (1); Hang, Calvin T (1); Chen, Hanying (2); Tsun, Zhi-Yang (1); Wu, Jiang (3); Shang, Ching (1); Bayle, J. Henri (3); Shou, W (2); Chang, Ching-Pin (1)
(1) Division of Cardiovascular Medicine, Stanford University School of Medicine (2) Department of Pediatrics, Indiana University School of Medicine (3) Department of Pathology, Stanford University School of Medicine

9:20 – 9:35 **Myocardial Smad4 Is Essential for Cardiogenesis in Mouse Embryos**
Song, Lanying (1); Yan, Wensheng (2); Chen, Xinbin (2); Wang, Qin (3); Jiao, Kai (1)
(1) Department of Genetics, Division of Genetic and Translational Medicine (2) Department of Cell Biology, (3) Department of Physiology and Biophysics, The University of Alabama at Birmingham, AL, USA

9:35 – 9:50 **Sonic hedgehog Modulates Addition of the Secondary Heart Field to the Arterial Pole**
Barbosky, Laura A.; Kirby, Margaret L
Department of Cell Biology, Duke University, Durham, NC

9:50 – 10:05 **BMP4 Function is Required in Second Heart Field-Derived Myocardium for Endocardial Cushion Remodeling, Outflow Tract Septation, and Semilunar Valve Development**
McCulley, David J (1); Kang, Ji-One (1); Agarwal, Pooja (1); Rojas, Anabel (1); Martin, James F (2); Black, Brian L (1, 3)
(1) Cardiovascular Research Institute and (3) Department of Biochemistry and Biophysics, University of California, San Francisco, California (2) Institute of Biosciences and Technology, Texas A&M Health Science Center, Houston, TX

10:05 – 10:20 **Retinoic Acid Deficiency Alters Secondary Heart Field Formation and NKx2.5 Regulation**
Ryckebusch, Lucile (1); Lin, Song-Chang (2); Wang, Zengxin (2); Chi, Xuan (3); Schwartz, Robert (3); Buckingham, Margaret (4); Zaffran, Stéphane (1); Niederreither, Karen (2)
(1) Developmental Biology Institute of Marseille-Luminy, France (2) Baylor College of Medicine, Houston, TX (3) The Institute of Biosciences and Technology, Houston, TX (4) Institut Pasteur, Paris, France.

10:20 am – 10:35 am **Break**

10:35 am – 12:10 pm **Platform Session VII**
Valvulogenesis
Chairs: Katherine Yutzey, Cincinnati Children's Medical Center and Andy Wessels, Medical University of South Carolina

10:35 – 10:40 **Chair's Introduction**

10:40 – 10:55 **Distinct Functions of the MAP3Kinases, MEKK3 and MEKK4, for Heart Valve Development**
Stevens, Mark V (1); Rogowitz, Elisa (2); Lalani, Sofia (3); Parker, Patti (1); Broka, Derrick (1); Vaillancourt, Richard R (1); Camenisch, Todd D (1, 4)
(1) Department of Pharmacology and Toxicology, University of Arizona (2) Department of Molecular and Cellular Biology, University of Arizona (3) Department of Cell Biology and Anatomy, University of Arizona (4) Sarver Heart Center, University of Arizona

10:55 – 11:10 **Tbx20 and Twist1 function in endocardial cushion mesenchyme**
Shelton, Elaine L; Yutzey, Katherine E
Molecular and Developmental Biology Graduate Program, University of Cincinnati.

11:10 – 11:25	**TGF-beta2 is required in vivo for epithelial-mesenchymal transformation, collagen fibrollogenesis, and differentiation, condensation and maturation of mesenchyme during valvulogenesis** *Azhar, Mohamad (1, 2); Yin, Moying (3); Martin, Jennifer (3); Pawloski, Sharon A; Prasad, Vikram (3); Rajan, Sudersan (3); Miller, Mariane (3); Chen, Dora (1); Runyan, Ray B.; Gittenberger-de Groot, Adriana C (4); Fuchs, Elaine (5); Doetschman, Tom (1, 2)* (1) BIO5 Institute, and (2) Dept of Cell Biology & Anatomy, University of Arizona, Tucson, AZ, (3) Dept of Molecular Genetics, University of Cincinnati College of Medicine, Cincinnati, Ohio, (4) Dept of Anatomy & Embryology, Leiden University Medical Center, Leiden, The Netherlands, (5) Howard Hughes Medical Institute, The Rockefeller University, New York, NY	
11:25 – 11:40	**The Role of Cartilage Link Protein 1 (Crtl1) in Heart Development** *Wirrig, Elaine; Snarr, Brian; Phelps, Aimee; O'Neal, Jessica; Barth, Jeremy; Kern, Christine; Fresco, Victor; Mjaatvedt, Corey; Hoffman, Stanley; Trusk, Thomas; Argraves, W. Scott; Wessels, Andy* The Medical University of South Carolina; Department of Cell Biology and Anatomy	
11:40 – 11:55	**Endocardial cells transformation is dependent on Par6 regulation of RhoA** *Townsend, Todd A (1); Wrana, Jeffrey L (2); Davis, George E (3); Barnett, Joey V (1)* (1) Department of Pharmacology, Vanderbilt University, Nashville, TN (2) Department of Medical Genetics and Microbiology, University of Toronto, Toronto, Ontario, Canada (3) Department of Medical Pharmacology and Physiology, Univ. of Missouri, Columbia, MO	
11:55 – 12:10	**NIH Presentation,** *Charlene Schramm, NHLBI*	
12:15 pm – 1:30 pm	**Lunch**	**Grand Hall Ballroom**
1:30 pm – 2:30 pm	**Business Meeting**	**Illinois Street Ballroom**
1:30 pm – 2:30 pm	**NIH Workshop** *Charlene Schramm*	**Edison Room**
2:30 pm – 2:45 pm	**Break**	
2:45 pm – 4:05 pm	**Platform Session VIII** **Animal Models of Congenital Heart Disease** *Chairs: Simon Conway, Indiana University and Christopher Brown, Vanderbilt University*	**Illinois Street Ballroom**
2:45 – 2:50	**Chair's Introduction**	
2:50 – 3:05	**A new mutant series provides insights into sensitivity of heart development to Tbx1-mRNA dosage.** *Zhang, Zhen; Baldini, Antonio* Center for Molecular Development and Disease, Institute of Biosciences and Technology, Health Science Center, Texas A&M University System.	

3:05 – 3:20 **Titration of Pax3 expression levels during morphogenesis of the outflow tract**
Zhou, Hongming; Conway, Simon J.
Cardiovascular Development Group, Herman B Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, IN

3:20 – 3:35 **Polycomb Repressive Complex I plays a cell autonomous role in cardiac development**
Jenkins, Caroline; Boutsma, Erwin; Bamforth, Simon; Joyce, Bradley; Franklyn, Angela; Broadbent, Carol; Schneider, Jurgen; Schwartz, Robert; Saga, Yumiko; Koseki, Haruhiko; van Lohuizen, Maarten; Bhattacharya, Shoumo
University of Oxford, Cardiovascular Medicine, Wellcome Trust Centre For Human Genetics

3:35 – 3:50 **Multiple functions of EIIIA and EIIIB splice isoforms of fibronectin in cardiovascular development.**
Astrof, Sophie (1); Hynes, Richard (2)
(1) Weill Medical School of Cornell University (2) MIT

3:50 – 4:05 **Loss of fibulin-1 in mice causes a DiGeorge syndrome-like phenotype**
Cooley, Marion A; Kern, Christine B; Fresco, Victor M; Wessels, Andy; Thompson, Robert P; McQuinn, Tim C; Twal, Waleed O; Mjaatvedt, Corey H; Drake, Christopher J; Argraves, W. Scott
Cardiovascular Developmental Biology Center, Department of Cell Biology and Anatomy, Medical University of South Carolina, Charleston, South Carolina, USA

4:05 pm – 4:30 pm **Break**

4:30 pm – 5:35 pm **Platform Session IX** **Illinois Street Ballroom**
Translational Models of Congenital Heart Disease
Chairs: Mark Payne, Indiana University and Jamie Lohr, University of Minnesota

4:30 – 4:35 **Chair's Introduction**

4:35 – 4:50 **A Novel Murine Model with Features of Vacterl and Caudal Regression Indicates a Common Genetic Origin for both Syndromes**
Szumska, Dorota (1); Pieles, Guido (1); Bilski, Michal (1); Franklyn, Angela (1); Cormack, Marie (3); Schneider, Juergen E (1); Jefferis, Joanna (4); Johnson, Paul (4); Lalanne, Zuzanna (3); Neubauer, Stefan (1); Clarke, Kieran (2); Brown, Steve D (3); Bhattacharya, Shoumo (1)
(1) Department of Cardiovascular Medicine, University of Oxford, UK (2) Department of Physiology, University of Oxford, UK (3) Mammalian Genetics Unit, MRC Harwell, UK (4) Nuffield Department of Surgery, University of Oxford, UK

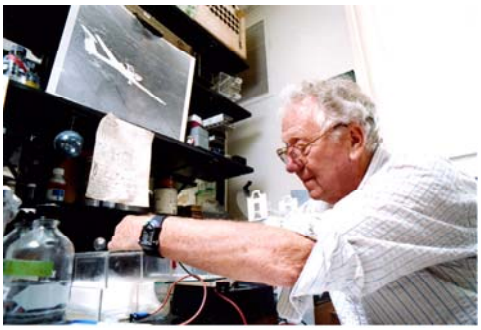
4:50 – 5:05 **Mediating ERK1/2 Signaling Rescues Congenital Heart Defects in a Mouse Model of Noonan Syndrome**
Nakamura, Tomoki; Colbert, Melissa C; Krenz, Maike; Molkentin, Jeffery D; Robbins, Jeffery
Division of Molecular Cardiovascular Biology, Cincinnati Children's Hospital Medical Center

5:05 – 5:20 **Muscleblind 2, a RNA binding protein important in Myotonic Dystrophy and Cardiac Conduction.**
Hao, Minqi; Akrami, Kevan; Wei, Ke; Vondriska, Thomas M; Tidball, James; Graves, Michael; Shieh, Perry B.; Chen, Fabian
University of California at Los Angeles, CA

5:20 – 5:35 **Duplication of the entire 22.9-Mb human chromosome 21 syntenic region on mouse chromosome 16 causes cardiovascular abnormalities**
Li, Zhongyou (1); Yu, Tao (1); Morishima, Masae (3); Pao, Annie (1); LaDuca, Jeffrey (1); Conroy, Jeffrey (1); Nowak, Norma (1, 2); Matsui, Sei-Ichi (1); Shiraishi, Isao (3); Yu, Y. Eugene (1, 2)
(1) Center for Genetics and Pharmacology, Roswell Park Cancer Institute, (2) New York State Center of Excellence in Bioinformatics and Life Sciences, (3) Department of Pediatric Cardiology and Nephrology, Kyoto Prefectural University of Medicine, Kyoto, Japan.

6:00 pm – 10:00 pm **Banquet (Open Bar) Grand Hall Ballroom**
Entertainment provided by the"Uptown Jazz Quartert"

KEYNOTE SPEAKERS



Oliver Smithies, D. Phil.
Excellence Professor of Pathology and Laboratory Medicine
The University of North Carolina at Chapel Hill

Oliver Smithies was born on June 23, 1925, in Halifax, England. He attended Oxford University and received a Bachelor of Arts Degree in Physiology with First Class Honors in 1946. In 1951, he obtained his M.A. and D.Phil. degrees in Biochemistry from Oxford. Smithies then moved to the United States as a Postdoctoral Fellow in Physical Chemistry at the University of Wisconsin. After two years at Wisconsin, Smithies accepted a position at the Connaught Medical Research Laboratories in Toronto and stayed there from 1953 to 1960, first as a Research Assistant and then as a Research associate. In 1960, Smithies returned to Wisconsin as Assistant Professor of Medical Genetics and Genetics, advancing to Full Professor by 1963. In 1989, Smithies moved to the University of North Carolina-Chapel Hill, where he is now the Excellence Professor of Pathology and Laboratory Medicine.

Early in his career, Smithies developed new methods for detecting genetic variation in proteins and originated starch gel as a supporting medium for the electrophoretic analysis of proteins and enzymes. This led to discoveries of protein polymorphisms and significant work on the heredity of important blood proteins – including haptoglobins, transferrins, and gamma globulins. This resulted in early recognition by the American Society of Human Genetics, who conferred upon him its William Allen Memorial Award in 1964.

Smithies continued his work on protein polymorphisms and the origins of antibody diversity. His many important contributions to genetics have been recognized by his colleagues: in 1971, he was elected a Member of the National Academy of Sciences; in 1975, he served a President of the Genetics Society of America; and in 1978, he became a Member of the American Academy of Arts and Sciences.

Smithies has also undertaken important national positions. He served as a Member of the National Advisory Medical Sciences Council for the National Institutes of Health from 1985 to 1990, helping establish research goals in the biomedical sciences.

He is one of only three people to twice receive the Gairdner Foundation International Award. This honor was awarded to smithies in 1990 “for the discovery, development and application of gel electrophoresis methods that allow the separation and identification of specific proteins and nucleic acids,” and in 1993 “for pioneering work in the use of homologous recombination to generate targeted mutations in the mouse.”

In 1988, when he joined the Department of Pathology at the University of North Carolina, Smithies was named Excellence Professor of Pathology. He remains an actively engaged scientist, working at the bench in his own laboratory, and has published more than 20 important papers over the last three years, in *Science, Nature, Cell, and Proceedings of the National Academy of Sciences*. His recent accomplishments have been in producing directed mutations in the mouse that mimic human genetic diseases or allow the dissection of complex genetic traits such as atherosclerosis and high blood pressure.

In recognition of his distinguished career, the University of Chicago conferred upon him the Honorary Doctor of Science Degree in 1991. The citation states, “innovator of concepts and technology in the fields of protein biochemistry, immunogenetics, molecular evolution and molecular biology, who has generated ideas and tools and used them to arrive at solutions to important biological problems and whose study of homologous recombination has laid the foundation for the rational use of gene therapy to correct genetic defects, representative of the highest ideal of the actively engaged scientist.”

Margaret L. Kirby, Ph.D.
Professor of Pediatrics, Cell Biology and Biology
Scientific Director, Neonatal-Perinatal Research Institute
Department of Pediatrics (Neonatology)
Duke University, Durham, NC

Margaret Loewy Kirby received her Ph.D. at the University of Arkansas for Medical Sciences at Little Rock. After a year in medical school she switched to research in anatomy under the guidance of Dr. Shirley Gilmore. Her dissertation research demonstrated that catecholamines are taken up by the developing notochord and included a detailed developmental history of the sympathetic trunks. Dr. Kirby received her A.B. degree in Biology from Manhattanville College, Purchase, NY, where her senior thesis focused on the effects of laser radiation on bacterial respiration.

After obtaining her PhD, Dr. Kirby taught at the University of Central Arkansas for two years and then accepted a postdoctoral fellowship with Dr. Alfred Heller at The University of Chicago. There her research showed the onset of norepinephrine synthesis in the sympathetic trunks. In addition, she developed a neonatal rat brain atlas, and she worked with Dr. Lloyd Roth on developing innervation of the eye. Because of the early synthesis of catecholamines with a long delay in functional innervation of target organs, she developed the hypothesis that these neural transmitters may be used in a different context in development.

Dr. Kirby accepted her first faculty position at the Medical College of Georgia where she continued work on developing autonomic innervation but now her research focused on this development in the heart where it was less complicated to analyze functional changes caused by pre-neural and neural transmitter functions. It was an attempt to prevent development of the parasympathetic innervation to the heart by ablation of the neural crest progenitors that led to her discovery of the cardiac neural crest which she determined to be required for normal septation and alignment of the outflow tract. In 1983 she published a seminal paper showing the requirement of neural crest in cardiac outflow tract development.

Dr. Kirby rose through the ranks at the Medical College of Georgia and in 1994 she was recognized for her research by being appointed Regents' Professor. With the help of an NIH program project grant, her work continued to focus on the role of neural crest in outflow alignment and her lab recognized a new role of neural crest cells in modulating signaling in the caudal pharynx concurrently with the discovery of the late contribution of outflow myocardium from the caudal pharynx that was regulated by signaling in the caudal pharynx.

Dr. Kirby retired from the Medical College of Georgia in 2001 and was recruited to the Department of Pediatrics (Neonatology) at Duke University where she continued to investigate the contribution of myocardium and smooth muscle to the arterial pole of the heart. Discovery of the genes controlling the migration and differentiation of these cells continues in both chick and zebrafish. Dr. Kirby has published 159 papers and 30 chapters and has recently authored a book entitled *Cardiac Development* (2007) published by Oxford University Press. Dr. Kirby's current research continues to focus on outflow development, subdivision of the cardiogenic fields and early heart tube formation, and the role of neural crest cells in controlling signaling in the pharynx that impacts early heart development.

CHARTER
Weinstein Cardiovascular Development Conference

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 a corporate memory and to maintain quality of the conference, the participants of the 1998 meeting voted to form an organizing committee called the "Weinstein Committee". The makeup of the committee is comprised of representatives from each of the three previous local organizing committees, representatives from the next two proposed meeting sites, and two "at large" members voted upon by the conference participants. The "at large" members will serve a three-year term. The charge to the Committee is to assist the local organizing committee with arrangements and organization and to help secure funding.

In addition, the Committee is charged with soliciting nominations for future meeting sites and hosts. Such nominations will then be brought up for a vote by the attendees during the business meeting. Meeting sites will be selected by vote such that the local organizing committee will have a two year lead-time. 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. 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. At this time participants will vote on future host and meeting site selection and may consider changes in the direction of the conference or its organization. At the 1999 meeting in Tucson, AZ this charter was distributed to the participants and ratified. Its provisions commenced at the business meeting of the 1999 Tucson, AZ conference.

The Charter will remain in effect until modified by a vote of participants at an annual business meeting.

WEINSTEIN 2007-CONFERENCE COMMITTEE

Weinstein Committee

Raymond Runyan, Ph.D. (Arizona, 2005)
Kersti Linask, Ph.D. (South Florida, 2006)
Loren Field, Ph.D. (Indiana, 2007)
James Martin, M.D., Ph.D. (Houston, 2008)
Brian Black, Ph.D. (San Francisco, 2009)
John Burch, Ph.D. (Fox Chase Cancer Center, at large)
Katherine Yutzey, Ph.D. (Cincinnati, at large)

Weinstein Local Organizing Committee Members, 2007

Loren Field, Ph.D., Indiana Universtiy
Mark Payne, M.D., Indiana University
Simon Conway, Ph.D., Indiana University
Anthony Firulli, Ph.D., Indiana University
Lei Wei, Ph.D, Indiana University
Weinian Shou, Ph.D., Indiana University
Michael Rubart, MD, Ph.D., Indiana University
Michiko Watanabe, Ph.D. Case Western Reserve University, Adjunct Member
Katherine Yutzey, Ph.D., Cincinnati Children's Medical Center, Adjunct Member

GENERAL ANNOUNCEMENTS

The Weinstein Cardiovascular Development Conference has become one of the most important meetings in the field of cardiovascular development. The informal setting, sharing of meal times, poster sessions, as well as other times for catching up with colleagues and making new collaborations have made this a favorite conference for many of us, both on the junior and senior researcher level. All of the oral presentations except for the Keynote lectures were selected, as in previous years, from the submitted abstracts.

Oral Presentations
Due to the number of topics that were chosen to be covered and the tight schedule to fit in as many talks as possible, each presentation is allotted 15 minutes for their presentation and questions. We request that the speakers adhere to the time limit.

Posters
Posters will be on display during the entire conference. Each poster has been assigned a unique number. Upon arrival, please mount your poster for display on the designated poster board number and area. The presenters of the even-numbered posters are requested to be at their poster during the Thursday evening poster session. The presenters of the odd-numbered posters are requested to be at their poster during the Friday evening poster session. Poster areas will be open and available throughout the entire conference up until 11:30 a.m. on Saturday for those of you who may wish to see posters that may have been missed earlier or for getting together with colleagues. A committee of judges will be circulating during the poster sessions during the times that presenters are expected to be at their poster to ask questions and to judge their poster presentation. The best graduate student and postdoctoral posters will be awarded prizes during the final dinner on Saturday.

Meals
All meals will be provided. There will be an open bar Thursday, Friday and Saturday evenings in the Grand Hall Ballroom from 6:00 pm to 10:00 pm.

Concurrent Workshops
Concurrent Workshops will be held on Thursday evening from 8:00 pm – 9:00 pm and Friday afternoon from 1:15 pm – 2:15 pm in the Edison Rooms. The workshops on Thursday include "Single v. multiple development heart fields" and "Cardiomyogenic stem cells during development". On Friday the workshops include "Cardiac neural crest" and "Imaging cardiovascular development and physiology".

NHLBI and NIEHS Presentations
Dr. Pat Mastin from the NIEHS will be speaking on Friday from 11:55 am – 12:00 pm, which is at the end of Platform Session IV. Dr. Charlene Schramm, from the NHLBI, will be speaking on Saturday from 11:55 am – 12:10 pm, which is at the end of Platform Session VII. Dr. Schramm will also have a NHLBI Workshop on Saturday from 1:30 pm – 2:30 pm in the Edison Room.

Thursday Evening
The Keynote presentation by Dr. Oliver Smithies will be followed by the first poster session & dinner buffet for all attendees.

Friday Evening
Following the Keynote by Dr. Margaret Kirby will be the second poster session and a buffet dinner.

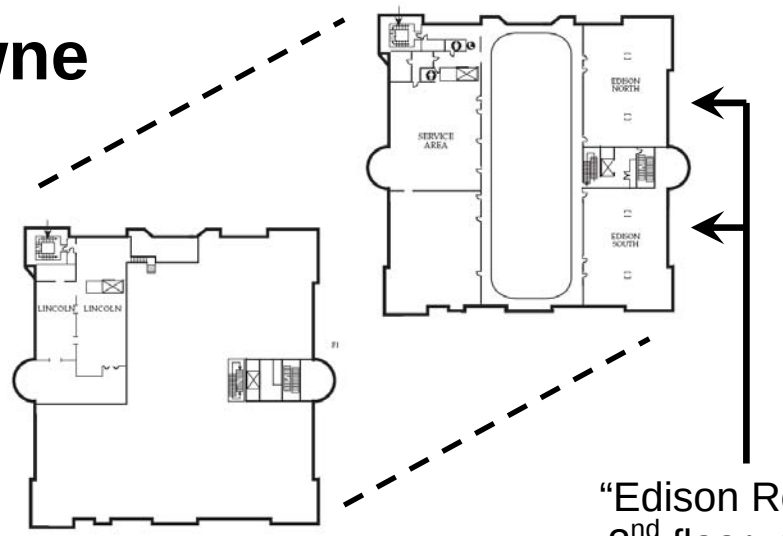
Business Meeting
The Weinstein business meeting will take place on Saturday from 1:30 p.m. - 2:30 p.m. in the Illinois Street Ballroom.

Saturday Evening (Banquet)
The Banquet on Saturday evening will take place in the Grand Hall Ballroom. Poster prizes will be awarded during the banquet. Entertainment for the evening will be provided by The Uptown Jazz Quartet. They have quite a repertoire!

Acknowledgements
We would like to thank Drs. Oliver Smithies and Margaret Kirby for agreeing to be our Keynote speakers this year. We also thank Dr. Constance Weinstein, who will be attending the 2007 Conference, for her support of cardiovascular development at NIH and providing the opportunity for these conferences.

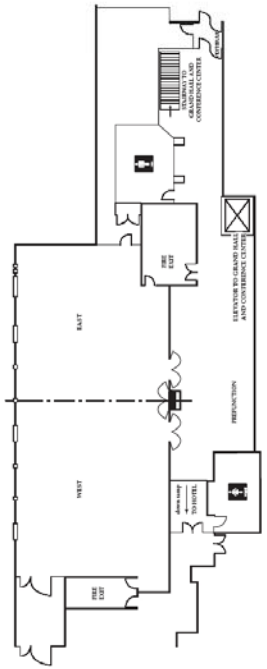
Many people beyond the local organizing committee have contributed to the organization of this conference. We are very thankful to everyone who has assisted in this year's event.

Union Station Crowne Plaza Hotel

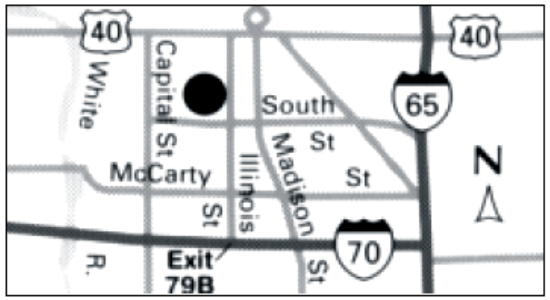


"Grand Hall Ballroom"
1st floor:
Dinners, drinks and posters

"Edison Rooms"
2nd floor, above the Grand Hall Ballroom:
Concurrent Workshops



"Illinois Street Ballroom"
2nd floor:
Lecture room for the platform presentations



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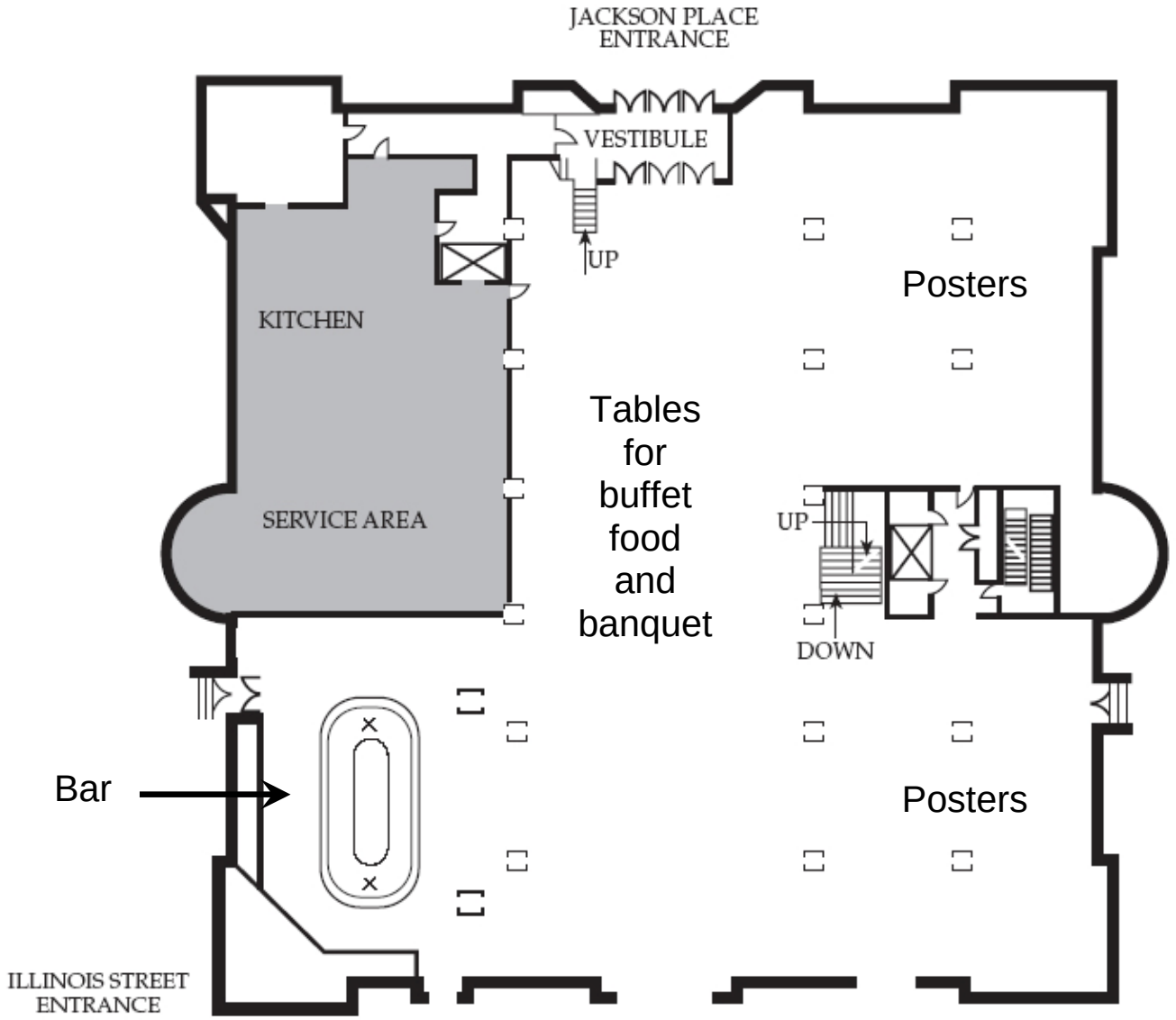
Grand Hall Ballroom: 1st floor



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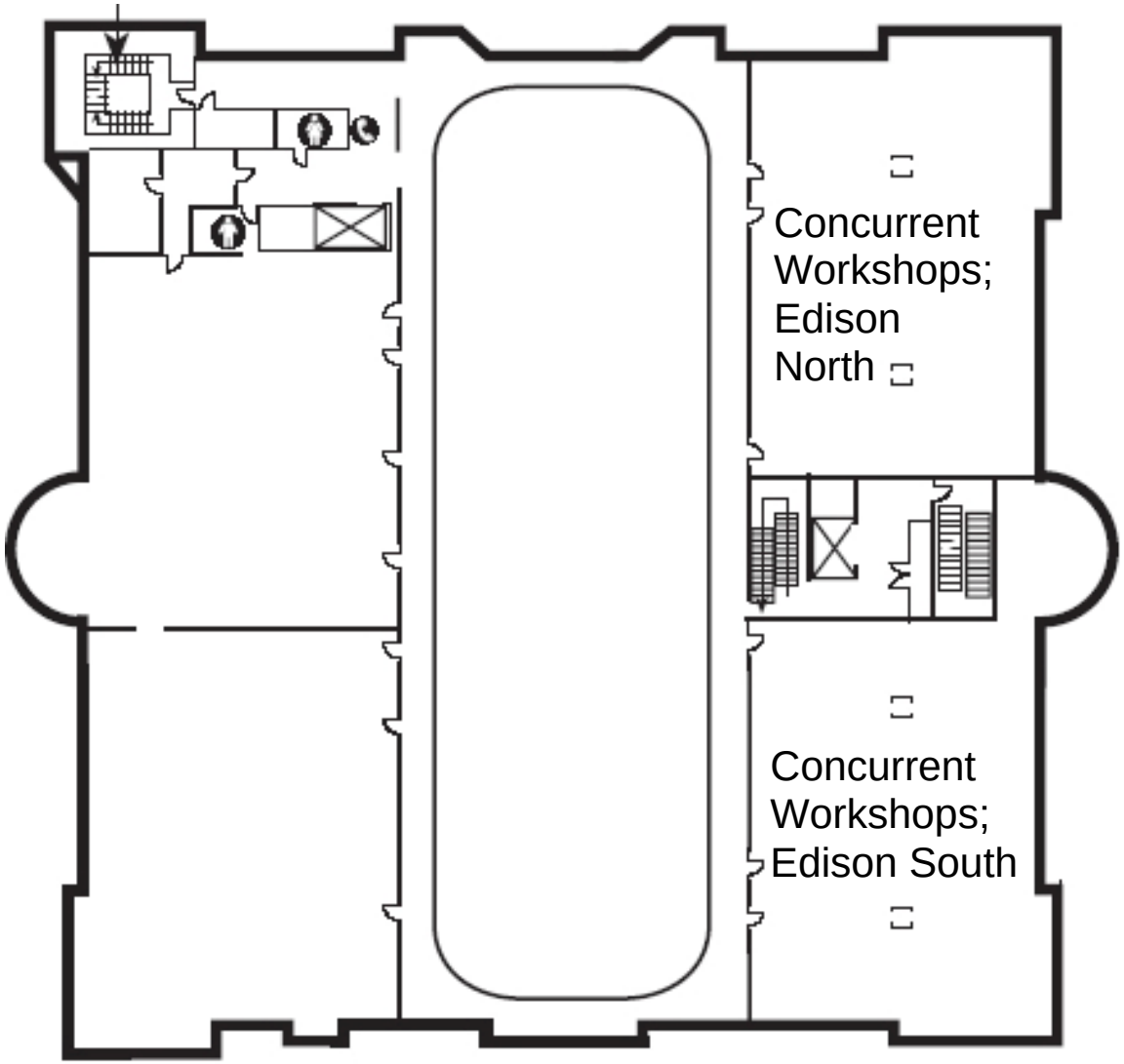
Edison Rooms, 2nd floor, above the Grand Ballroom



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CONFERENCE SUPPORTERS

We gratefully acknowledge the following organizations and companies for their generous support for this conference. This support helped maintain registration at affordable levels and to provide for a substantial number of travel grants to junior investigators.

This project was supported (in part) by NIH Research Grant #R12H174373 funded by the **National Heart, Lung and Blood Institute**, the **National Institute of Child Health and Human Development**, the **National Institute of Environmental Health Sciences**, and the **Office of Rare Disease**. (The views expressed in written conference materials or publications and speakers and moderators do not necessarily reflect the official policies of the Department of Health and Human Services; nor does mention of trade names, commercial practices, or organizations imply endorsement by the U.S. Government.)

Supported by an educational grant from **Eli Lilly & Company.**

Travel Awards are sponsored in part by the **American Association of Anatomists.**

American Heart Association

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Department of Pediatrics
Section of Pediatric Cardiology
Wells Center for Pediatric Research
Department of Medicine
Krannert Institute of Cardiology

PLATFORM PRESENTATION ABSTRACTS

Platform Session I

1. Cardiomyocyte and Epicardial Cell Addition during Cardiac Homeostasis in Adult Zebrafish
Wills, Airon A; Holdway, Jennifer; Major, Robert J; Poss, Kenneth D
Duke University Medical Center, Dept. of Cell Biology

The heart maintains structural and functional integrity during years of continual contraction and physiologic change, but the extent to which new cell addition participates in cardiac homeostasis is unclear. Here, we assessed cellular and molecular mechanisms of cardiac homeostasis in zebrafish, animals that grow as adults and possess an unusual capacity to regenerate after acute cardiac injury. During rapid animal growth, robust hyperplastic cardiomyogenesis was observed throughout the growing ventricle. By contrast, new cardiomyocyte generation was present, albeit rare, in ventricles of animals maintaining size. We also observed robust supplementation of the ventricle wall with epicardial-derived cells during rapid animal growth, greater than that seen in animals growing slowly or maintaining size, and comparable to that observed during regeneration. Growth-stimulated epicardial gene expression could be reproduced by manipulating the extra-cardiac environment, suggesting that the epicardium perceives extra-cardiac space available for growth. Inhibition of Fgf signaling, a pathway required for normal heart regeneration, disrupted epicardial supplementation during growth and caused spontaneous ventricular scarring in animals maintaining size. Together, these results reveal mechanisms that stimulate myocardial cell creation and epicardial supplementation proportionally to couple ventricular size and function with animal size.

2. FOXO transcription factors in the regulation of cardiac myocyte proliferation and myocardial growth during development
Evans-Anderson, Heather J; Alfieri, Christina M; Yutzey, Katherine E
Division of Molecular Cardiovascular Biology, Cincinnati Children's Hospital Medical Center

The regulatory mechanisms of cardiac myocyte proliferation and resultant myocardial growth are not well defined. Current data suggests Forkhead Box Other (FOXO) transcription factors may have a significant role in the mediation of myocardial growth during development. FOXO factors are antagonized by AKT via phosphorylation, which results in nuclear export and inactivation. In the absence of active AKT, FOXO factors can negatively regulate proliferation through induction of the Cip/Kip family of cyclin kinase inhibitors. Thus, FOXO factors could provide a direct mechanism to control the cardiac myocyte cell cycle downstream of PI3K/AKT signaling. Preliminary data show that FOXO1 and FOXO3 are expressed in the developing myocardium from embryonic to neonatal stages consistent with decreasing rates of myocyte proliferation. Expression of p21cip1, a FOXO target gene and cell cycle inhibitor, in mature trabecular myocytes coincides with FOXO1 nuclear localization and decreased myocyte proliferation during the final stages of heart development. In vitro studies show that cultured embryonic, fetal, and neonatal cardiac myocytes are responsive to IGF1 stimulation, which results in the induction of the PI3K/AKT pathway, phosphorylation of FOXO proteins, and increased myocyte proliferation. Likewise, adenoviral-mediated expression of AKT promotes cardiac myocyte proliferation and increased cell size. In contrast, increased expression of FOXO or FOXO target genes negatively affects myocyte proliferation and growth. In vivo cardiac-specific expression of FOXO1 during heart development causes embryonic lethality at E10.5 due to severe myocardial defects. Alternatively, dominant negative FOXO1 transgenic embryos appear normal at E10.5, but display abnormal morphology of the myocardium by E18.5. These data support FOXO transcription factors as downstream effectors of PI3K/AKT signaling in the negative regulation of cardiac myocyte proliferation and myocardial growth. Current studies include comparative analysis of the effects of FOXO gain and loss of function to identify FOXO transcriptional mechanisms of cell cycle regulation during heart development.

3. 3D Visualization of proliferation in cardiac and extracardiac mesoderm
van den Berg, G; Soufan, AT; de Boer, PAJ; van den Hoff, MJB; Moorman, AFM
Heart Failure Research Centre, Amsterdam, The Netherlands

Proliferation is a crucial contributor to organogenesis, but its regionalization during heart development is poorly understood. Recently we demonstrated that after initial formation of the heart tube, proliferation in the myocardium ceases. Nonetheless, the number of cardiomyocytes increases. This is because cells are still being added to the myocardial lineage. To further analyze this process, we studied proliferation (as defined by the nuclear fraction that is positive for BrdU after 1 hour of exposure) in the embryonic chicken heart and its contiguous mesoderm. The pattern of proliferation is presented three-dimensionally in order to provide a comprehendible image of its role in the intricate morphogenesis of early heart formation. We show that upon fusion of the mesoderm in the midline, which marks the initial formation of the heart tube, there is no proliferation. However, the mesoderm that is contiguous to the forming heart, and which lines the embryonic coelom, shows high proliferation. Later in development, myocardium has formed and can be distinguished as a trough. This myocardium does not proliferate. The mesoderm which is located laterally and dorsally hardly proliferates, whereas the caudally located coelomic wall proliferates extensively. Later in development this pattern is still observable, but now dorsally the non-myocardial mesoderm can be divided into a non-proliferating venous inflow and a highly proliferating coelomic wall. During further development working myocardium of the embryonic ventricle starts to proliferate at the outer curvature. Proliferation outside the heart has extended cranially, along the dorsal wall of the coelom, towards the myocardial outflow tract. Our data show a non-proliferating, but nonetheless growing myocardial heart tube. Addition of cells occurs from the flanking and highly proliferating mesoderm. Early in development a zone of high proliferation is located dorsally to the forming myocardium. This zone lines the coelomic cavity and with time extends cranially towards the outflow of the heart. These data suggest that this zone can contribute cells to both inflow and outflow myocardium. Also, these data show the coelomic wall to be an important structure in organogenesis.

4. Canonical Wnt Signaling is Required for Mammalian Cardiogenesis by Regulating Cardiac Progenitors
Kwon, Chulan; Arnold, Joshua; Taketo, Makoto; Srivastava, Deepak
Gladstone Institute, UCSF

Guiding stem or progenitor cells into distinct lineages and controlling their expansion remain fundamental challenges in stem cell biology. Members of the Wnt pathway control many pivotal embryonic events1-3, often regulating self-renewal or expansion of progenitor cells. Canonical Wnt ligands are thought to negatively regulate cardiomyogenesis in several species4-6. However, the cell-autonomous role of canonical Wnt signaling through its obligatory transcriptional mediator, b-catenin, is unknown. We used tissue-specific in vivo genetic manipulation to show that b-catenin is an essential positive regulator of proliferative expansion in cardiac progenitor cells. The Wnt/b-catenin target gene cyclin D2 was downregulated in hearts lacking b-catenin and upregulated in hearts with stabilized b-catenin. At discrete windows of development in embryonic stem cells, activation of canonical Wnt signaling promoted expansion of cardiac progenitors after initial commitment, and inhibition of canonical Wnts repressed further cardiac differentiation. Thus, canonical Wnt signaling promotes the expansion of cardiac progenitors and differentiation of cardiomyocytes.

Platform Session II

5. MEF2A controls a costameric network of genes in cardiac muscle
Naya, Frank J; Brand, Ondra M; Reynolds, Joseph G; McCalmon, Sarah A
Department of Biology, Program in Cell and Molecular Biology, Boston University

The MEF2 transcription factor family plays an important role in cardiac muscle development and disease by regulating a vast array of downstream target genes. These target genes can be activated by multiple MEF2 factors in vitro. Knockout studies in mice, however, have revealed remarkably different

phenotypes associated with loss-of-function of the individual MEF2 factors. MEF2A knockout mice display perinatal cardiac sudden death with myofibrillar and conduction abnormalities which is in striking contrast to the cardiac looping morphogenesis defects observed in MEF2C knockout embryos. Although the MEF2 factors are co-expressed in muscle, these observations suggest that individual MEF2 proteins function at different times in development by regulating a specific class of target genes. To identify genes dependent on MEF2A transcriptional activity gene expression profiling was performed on MEF2A-deficient hearts. Two novel MEF2A-dependent genes, myospryn and myomaxin, were identified that encode scaffolding proteins localized to the muscle costamere, an elaborate protein network that connects the muscle plasma membrane to the contractile apparatus at the periphery of the Z-disc. The importance of the costamere is exemplified by perturbations in this structure in various muscle disorders resulting from mutations in resident costameric proteins. The localization of these novel MEF2A targets to the costameric region of muscle prompted us to examine a large cohort of genes comprising the myofibrillar-costameric network of proteins. While many muscle structural genes harbor putative MEF2 binding sites, to our surprise, only a specific subgroup of genes were down-regulated in mef2a knockout hearts. Thus, despite the broad spectrum of genes bound by the MEF2 transcription factor family our findings indicate that MEF2A controls the expression of a restricted subset of genes that localize to the costameric region of muscle. These studies implicate MEF2A in the control of myofibrillar assembly during development and maturation of cardiac muscle.

6. Investigating the role of Prox1 in the developing heart

Risebro, Catherine A (1); Searles, Richelle G (1); Melville, Athalie AD (1); Ehler, Elisabeth (2); Harvey, Natasha L (3); Dillard, Miriam (4); Moses, Kelvin (5); Schwartz, Robert J (5); Chien, Kenneth R (6); Oliver, Guillermo (4); Riley, Paul R (1)
(1) UCL Institute of Child Health, London, England (2) The Randall Centre of Cell and Molecular Biophysics, Guy's Campus, Kings College, London, England (3) The Hanson Institute, Adelaide, Australia (4) St. Jude Children's Research Hospital, Memphis, USA (5) Texas A&M University Health Science Center, Houston, USA (6) MGH Cardiovascular Research Center, Boston, USA

The prospero-related homeobox transcription factor, Prox1, has been implicated in the development of multiple tissues in the mouse including the eye, liver, pancreas and lymphatic vasculature. In zebrafish, Prox1 is necessary for myofibrillar organisation, maturation and differentiation of slow-twitch skeletal muscle fibres. In the mouse, Prox1 is expressed throughout the myocardium of the developing heart, suggesting that Prox1 functions in multiple muscle lineages. Analysis of both conventional and cardiac-specific conditional knockouts has revealed that Prox1 is an essential regulator of mouse cardiac development. In Prox1-deficient myocardium the myofibrils are highly disorganised and have severely disrupted Z-discs from E12.5. These results suggest that Prox1 regulates the maintenance and/or maturation of cardiac muscle ultrastructure. As such, Prox1 is one of the first transcription factors identified to play such a role. Furthermore, Prox1 is involved in endocardial cushion formation and impacts upon subsequent valve and interventricular septum formation. ChIP-on-chip is being performed to identify definitive downstream targets of Prox1 to gain insight into the mechanisms by which Prox1 exerts its effect on the developing myocardium and valves of the heart.

7. High resolution in vivo imaging of the cross-sectional deformations of contracting embryonic heart loops using optical coherence tomography (OCT)

Manner, Jorg (1); Thrane, Lars (2); Norozi, Kambiz (3); Yelbuz, Mesud T (3)
(1) Department of Anatomy and Embryology, Georg-August-University of Göttingen, Germany (2) Optics & Plasma Research Department, Risø, Denmark (3) Department of Pediatric Cardiology and Intensive Care Medicine, MHH Hannover, Germany

The early embryonic heart is a simple tube-like organ that propels the blood from its venous to its arterial end by peristaltoid contractions. It is usually thought that the endocardial lumen of the early heart tube is circular in cross section during the whole contraction cycle. In the present study, we have analyzed, for the first time, the cyclic deformations of contracting embryonic heart tubes using real time high resolution optical coherence tomography (OCT). Studies were carried out on chick embryos (HH-9 to HH-18) in

shell-less culture. Our data show that the endocardial lumen of the early heart tube (HH-9 to Hh-12) is not circular but merely spindle-shaped in cross section at the end of diastole. During contraction, the endocardial lumen becomes obliterated by the deformation of the spindle-shaped lumen into a slit-like lumen. Our results show that the peristaltoid contractions of the early embryonic heart run in a different way than originally thought.

8. Mouse mutants with defects in cardiovascular development isolated from balancer chromosome mutagenesis screens

Mitchell, Karen (1);Risley, Michael D (1);Green, David (1);Justice, Monica J (2); Hentges,Kathryn E (1)
(1) Faculty of Life Sciences, The University of Manchester, Manchester, England M13 9PT, (2) Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas, USA

Region-specific mutagenesis screens can provide a functional annotation of a genomic interval by identifying the phenotypes that are caused by mutating genes in that region of the genome. Two mouse balancer chromosome mutagenesis screens, for regions of mouse chromosomes 4 and 11, have been performed. These screens allowed the isolation of lethal mutations in the balancer region without bias towards any phenotype or stage of death. Analysis of mutant embryos at several developmental stages revealed many mutant lines with defects in cardiovascular development. One mutant line isolated from the chromosome 11 balancer screen, L11Jus27, has an enlarged heart and delayed development. Cardiac muscle markers are abnormal in these mutant embryos, suggesting defects in myogenesis. Mutant hearts also do not beat normally when observed during dissection. There is abnormal vascular development in both the mutant embryo and the yolk sac. Another mutant line, L11Jus8, has abnormal yolk sac vasculature, defective cardiac muscle development, and haemorrhage leading to death after mid-gestation. Further characterisation of the mutants will identify the developmental processes that are regulated by the L11Jus8 gene, and advance our understanding of cardiovascular development. Several other cardiovascular mutants were isolated in the balancer chromosome mutagenesis screens. Some of the phenotypes observed include excessive embryonic vasculature, heart development outside the amniotic membrane, and embryos with abnormal yolk sac appearance suggesting defects in vascular remodeling. Because the mutagenesis screens employed balancer chromosomes, all the mutations are already mapped to the balancer interval. Many of the mutant phenotypes isolated in the balancer screen are distinct from previous mutants that map to these regions of the genome. The mutants isolated from these balancer chromosome screens provide a functional annotation of mouse chromosomes 4 and 11, and demonstrate that many novel developmental phenotypes can be quickly isolated in defined genomic intervals through balancer chromosome mutagenesis screens.

Platform Session III

9. Histone deacetylase 1(HDAC1) is Essential for Cardiac Development in Zebrafish

Zchut, Sigalit (1); Warren, Kerri S (2); Creton, Robbert (3); Kochilas, Lazaros (1)
(1) Pediatric Cardiology, Department of Pediatrics, Rhode Island Hospital Providence, RI (2) Department of Biology, Roger Williams University, Bristol, RI (3) Department of molecular Biology, Cell Biology and Biochemistry, Brown University, Providence, RI

Histone deacetylases (HDACs) are a family of conserved proteins that regulate transcription by affecting chromatin remodeling. In zebrafish hdac1 deficient embryos display a diminutive and severely dysplastic heart with decreased heart rate, pericardial effusion and ineffective circulation. To examine when and how hdac1 functions in heart development, we examined the expression of markers of the developing cardiac primordial. Although wild type (wt) and hdac1 mutant embryos appear identical in their expression of hand2 in the anterior lateral plate mesoderm at the 10s stage, there is significant delay in the expression of nkx2.5 between 10s and 21s stages. This results in delayed myocardial differentiation as reflected by the delayed expression of cmlc2 and vmhc in the developing heart. As development of the cardiac field progresses it is evident that the hdac1 mutant cardiac field is smaller and has fewer cmlc2 and vhmc positive cells in comparison with the wt. Also, the heart tube of the hdac1 mutant embryo fails to extend and the atrium fails to coalesce and maintains a funnel shape. Tbx5 and bmp4 have also a

markedly reduced expression level in the cardiac tube, although they maintain a normal expression pattern in other tissues such as the fin buds, the retina and the ear at 26 hpf. Crossing the hdac1 mutants with the fli1-egfp line reveals no apparent vascular defects at the time of blood flow initiation in the hdac1 deficient embryos, however at a later stage some major blood vessels fail to develop, such as the ducts of Couvier, the brancial arches and certain cranial vessels. In conclusion, our studies indicate that hdac1 is required for cardiac development in zebrafish. More specifically, our results demonstrate that hdac1 plays an important role in early cardiomyocyte differentiation and expansion of the cardiac field.

10. In search for partners of Tbx2 and Tbx3

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Abstract: T-box transcription factors Tbx2 and Tbx3 are important regulators in the development of the heart. They have been shown to play crucial roles in the development of the sinoatrial node, atrioventricular canal (AVC) and outflow tract. In these structures Tbx2 and Tbx3 repress a cardiac chamber-specific gene program, including connexin 40 (Cx40), Cx43 and atrial natriuretic factor (ANF), preventing the cells to differentiate into working myocardium. However, the precise mechanisms by which Tbx2 and Tbx3 exert their functions remains unclear, and may involve cofactors that provide specificity and function to these factors. Therefore we are currently using several approaches to identify the proteins that interact with Tbx2 and Tbx3 during heart development. Using a yeast-2-hybrid screen and concurrent protein-pull down assays, we have shown that Tbx2 and Tbx3 interact with homeobox proteins Msx1 and Msx2. Msx1 is expressed in the mesenchyme of the AV-cushions whereas Msx2 is more abundant in the myocardial layer of the AVC, clearly overlapping with both Tbx2 and Tbx3. We show that Msx1 and Msx2 can repress the expression of ANF and Cx43 in a heart derived cell-line [H10] and that knockdown of Tbx3 relieves repression of Cx43 by Msx-proteins. In vitro promotor binding studies show that Msx1 and Msx2 interact with several sites in a 700bp minimal ANF promotor. ChIP experiments on cell-lines and heart tissue are being undertaken to validate these results. Furthermore, we are currently analyzing Tbx2-Msx2 and Tbx3-Msx2 compound mutant embryos to identify the functional role of this interaction in heart development. In parallel, we are setting up a system to isolate whole protein complexes from mammalian cells. Therefore, we have created a cell-line that expresses Tbx3 with two affinity tags. Since these cells express the fusion protein at a near to endogenous level, Tbx3 can form protein complexes with physiological interacting proteins. Although we are still optimizing the procedure, we show the successful isolation and dual affinity purification of the tagged Tbx3.

11. Cooperative function of the transcription factors Nkx2.5 and Mef2c during heart development

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The molecular programs employed to affect the developmental events that transpire during the formation of the mammalian heart are orchestrated by the interactions and activities of a diverse profile of transcription factors. Among these, the homeobox transcription factor Nkx2.5 and the MADS-box transcription factor Mef2c elicit striking phenotypic similarities when disrupted, and regulate the expression of a number of common genes. These functional parallels raise the possibility that Nkx2.5 and Mef2c genetically and molecularly interact during heart development. To test this, we have assessed the cardiac phenotype of Nkx2.5-/-;Mef2c-/- doubly mutant embryos. Although heart development in both Nkx2.5-/- and Mef2c-/- mutants arrests during looping, both individual mutants have morphologically and molecularly identifiable ventricles. However, histological and molecular analyses indicate that Nkx2.5-/-;Mef2c-/- double mutants display ventricular hypoplasia, a more severe cardiac phenotype than those associated with either single mutant. These data support a genetic interaction between Nkx2.5 and Mef2c. Additionally, both co-immunoprecipitation experiments employing epitope-tagged proteins and mammalian 2-hybrid analyses have suggested a molecular interaction between Nkx2.5 and Mef2c. To illustrate the physiological significance of these genetic and molecular interactions, we have undertaken a

candidate gene approach, identifying genes regulated by both Nkx2.5 and Mef2c, such as atrial natriuretic factor (ANF). Luciferase experiments using the ANF promoter have supported the hypothesis that Nkx2.5 and Mef2c synergize to upregulate transcription. We are currently performing additional gel shift and luciferase experiments to determine whether Nkx2.5 and Mef2c directly cooperate to trans-activate other target genes. The results obtained from these studies will serve to define the functional role of Nkx2.5 and Mef2c interactions during cardiovascular development and potentially provide insight into human congenital disease.

12. Nkx Genes Regulate Heart Tube Extension in Zebrafish

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The cardiac homeodomain transcription factor Nkx2-5 is a key causative gene associated with congenital heart disease in humans. Previous studies in mouse have shown that Nkx2-5 is essential for cardiac chamber morphogenesis and myocardial maturation. Although Nkx2-5 clearly plays a fundamental role in cardiac development, we do not understand how it regulates the underlying cellular mechanisms that guide cardiac chamber formation. With the benefit of a transparent zebrafish embryo, we can combine embryologic and genetic approaches to analyze cardiac cell behavior during morphogenesis. The zebrafish genome contains two Nkx2-5 homologs that are expressed in cardiomyocytes, nkx2.5 and nkx2.7. Here we show that nkx2.5 and nkx2.7 play a previously unappreciated, crucial role in the process of heart tube extension. Injection of anti-nkx2.5 and anti-nkx2.7 morpholinos disrupts cardiac morphogenesis, leading to the formation of a small, misshapen ventricular chamber and an enlarged, bulbous atrial chamber. Morphogenetic defects are first evident during the earliest stages of heart tube assembly. In wild type embryos, a critical transition occurs as bilateral cell populations fuse and extend into a long and narrow heart tube. In contrast, nkx-deficient embryos have an abnormally short and wide ventricular portion of the heart tube and a disorganized, sprawling atrial component. As a first step toward assessing the cellular mechanisms underlying this phenotype, we determined that chamber specific precursor cell populations are established normally in nkx-deficient embryos. However, as morphogenesis proceeds, the loss of nkx gene function leads to a mild reduction in the number of ventricular cardiomyocytes and a surplus of atrial cardiomyocytes. Therefore, we conclude that nkx genes play multiple roles in guiding heart tube extension through differential effects on ventricular and atrial cell number.

13. DPF3 – a new key transcription factor – bridging chromatin remodeling and cardiac muscle specification

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The transcription factor DPF3 occurring in two splice forms, containing zinc- and half/double PHD-fingers, first came to our interest by being differentially expressed in cardiac tissue of patients with Tetralogy of Fallot. Taking a systematic approach including methods such as in situ hybridization, electron-microscopy, gene expression arrays, siRNA knock-down, morpholino treatment, reporter gene assays, EMSA, chromatin-immunoprecipitation with array detection (ChIP-chip), GST pull-down and TAP-tag, we were able to elucidate the function and role of DPF3 in cardiomyocyte specification. DPF3 shows evolutionary conserved cardiac expression at early stages of embryonic development from mouse to zebrafish, and is a direct target of Nkx2.5. DPF3 protein recognizes and interacts - in an isoform-specific-manner - with specifically modified histone tail residues H3K4me3 and H3K9ac, H3K14ac. Thus it represents the first protein of its class identified with such function. Morpholino-mediated knock-down of dpf3 disrupts heart chamber specification, sarcomere assembly and leads to a loss of ventricular

contractility in zebrafish. Morphants show a deregulation of myofibrillar proteins, regulators of sarcomere assembly such as cyma1 (cardiomyopathy associated1), actin capping protein capZ as well as cardiac transcription factors, id2, irx4a and hand2. id2 is also a direct target of DPF3 and potentially represents the key target initiating the down-stream circuit leading to the observed phenotype. Loss- and gain of function studies in mouse and zebrafish have shown that cardiac chamber specification is regulated by a hand2/irx4-dependent pathway and that perturbations to this pathway lead to ectopic expression of chamber-specific markers such as observed in DPF3-morphants. Considering the above, our original observation of upregulation of DPF3 in patients may reflect the turn-on of the embryonic specification program by cardiac adaptation to abnormal physiology.

Platform Session IV

14. Connexin43 in the Epicardium is Required for Normal Coronary Development

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Connexin43 (Cx43) knockout mice exhibit anomalous coronary vessel development accompanied by the formation of infundibular pouches. These abnormal pouches result in the obstruction of the pulmonary outflow tract and ultimately, death of the mice at birth. These pouches contain ectopic vascular smooth muscle, endothelial cells and fibroblasts. All three of these cell lineages are epicardially derived and thus, strongly suggest a role for epicardial derivatives in the cardiac anomalies in the Cx43 KO mouse. In this study, we show significant Cx43 deficiency leads to a change in epicardial cell morphology with perturbation in the organization of the actin cytoskeleton and focal adhesion contacts. Motion analysis to examine epicardial cell motile behavior revealed altered cell protrusive activity accompanied by a reduction in directionality but an increase in the speed of cell locomotion. Using a 3-dimensional collagen gel assay, we showed an enhanced epithelial-mesenchymal cell transition (EMT) in the Cx43 KO epicardium, a process crucial in the production of coronary vascular progenitors. This change in EMT was accompanied by an increase in the expression of vascular endothelial growth factor (VEGF). Whole mount PECAM staining of the embryonic heart showed altered patterning of the coronary vascular plexuses and defects in the remodeling and maturation of the coronary vascular tree. The expression of a number of other genes associated with EMT and coronary vasculogenesis were also altered, including members of the Tgfβ and Fgf families. These findings demonstrate an important role for Cx43 in coronary vasculogenesis, and suggests this may involve a role in the regulation of epicardial EMT.

15. Endothelial-Specific Ablation of Serum Response Factor Results in Vascular Instability and Embryonic Lethality

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Serum response factor (SRF), a nuclear transcription factor member of the MADS box family, plays an important role in cardiovascular development and function. Significant work demonstrates a central role for SRF in regulating smooth and cardiac muscle cell gene expression. Consistent with this, loss of SRF function blocks differentiation of vascular smooth muscle cells from the proepicardial precursors, indicating SRF is necessary for coronary vasculogenesis. The role of SRF in endothelial cell contribution during early vascular development, however, has not been addressed. To investigate this, we generated transgenic mice lacking expression of SRF in endothelial cells. Mice expressing Cre recombinase [Cre+] under Tie2 promoter control were bred to mice homozygous for SRF alleles containing loxP recombination sites within the SRF gene [Srf(f/f)]. Tie2 is a tyrosine kinase receptor expressed predominantly on endothelial cells. Along with the related receptor Tie1, it mediates signaling during different stages of blood vessel remodeling. Resulting embryos were harvested at specific ages for observation of physical condition and analysis of genotype. Tie2·Cre+/Srf(f/f) embryos appeared to develop normally compared to wild-type littermates until embryonic day 10.5 (E10.5). Beginning at E11.5, Tie2·Cre+/Srf(f/f) embryos exhibited cerebrovascular hemorrhaging and severely disrupted

vascular networks within the yolk sac. Hemorrhaging in mutant embryos became more generalized with progressive age, and by E15.5, all Tie2·Cre+/Srf(f/f) embryos observed were nonviable and/or resorbing. Initial immunohistochemical analysis revealed apparently normal vascular endothelial cell differentiation; however, newly developed vessels fail to undergo appropriate remodeling. These results provide the first in vivo evidence in support of a role for SRF in endothelial cell function and strongly suggest SRF plays an important role in appropriate vascular remodeling.

16. VEGF-A164 regulates coronary endothelial proliferation, but not tubulogenesis

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The coronary vascular system develops from mesothelial cells called the proepicardium (PE) that project from the septum transversum. Coronary development is initiated as a subset of epicardial cells undergo an epithelial to mesenchymal cell transformation (EMT) and migrate into the extracellular matrix-rich subepicardial space and undergo vasculogenesis and angiogenesis in a process termed tubulogenesis. It has been established that the epicardium stimulates cardiac myocyte proliferation. Less is known about how the myocardium effects coronary development. To address this we have generated an in vitro model in which PE cells undergo EMT and differentiate into capillary-like networks of endothelial cells (Nesbitt et al, 2007). Co-culturing PE cells with cardiac myocytes on the scaffold significantly increased both endothelial cell (EC) density and the tubulogenesis. VEGF-A164 was investigated as a candidate molecule for regulating coronary tubulogenesis because of previous studies and is expressed by the cardiac myocytes in our cultures. Our studies found that PE-derived ECs proliferated robustly when cultured on scaffolds containing VEGF-A164. In fact, ECs in these cultures had proliferation indexes that were significantly higher than PE/cardiac myocyte co-cultures. However, no endothelial tubes were found in these cultures. Thus, VEGF-A164 appears to mediate EC proliferation but not morphogenesis into coronary vasculature. When the VEGF receptor, Flk-1, was inhibited both EC proliferation and tubulogenesis was significantly down regulated in comparison to controls. These data indicate that VEGF signaling is critical to both coronary EC proliferation and tubulogenesis. Future studies include the investigation of other VEGF family members to determine their roles in coronary development. The 3-D tube model has proven to be a useful tool in delineation of the molecular, and cell-cell interactions that regulate cardiac development. We have also used this model recently to investigate the role of mechanical forces, such as fluid flow in coronary vessel maturation.

17. Canonical Wnt Signaling in Endothelial Cells is Essential for Central Nervous System Vascularization and Blood-Brain Barrier Development

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The molecular mechanisms controlling the coordinated development of an organ and its vasculature are poorly defined. Also, the type of endothelium formed depends on the nature of the organ. In the mouse, the central nervous system (CNS) vasculature forms by angiogenesis from the surrounding perineural vascular plexus (PNVP) and becomes highly specialized forming a tight barrier between the blood and the CNS, the blood-brain barrier (BBB). Using the developing CNS as a model system, we unexpectedly found that Wnt ligands produced by the developing neuroepithelium act directly on the endothelium of the PNVP via the canonical pathway to both control CNS vascularization and establish properties of the BBB.

18. Effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on hypoxia-inducible factor-1 alpha during chick cardiogenesis

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Differential tissue hypoxia drives normal cardiogenic events including coronary vessel development. We tested whether this requirement renders cardiogenic processes susceptible to teratogens that activate a transcriptional pathway that intersects with the hypoxia inducible factor (HIF-1) pathway. The toxin TCDD is known to cause cardiovascular defects by way of reduced myocardial hypoxia, inhibition of angiogenic stimuli and the responsiveness of endothelial cells to those stimuli. Our working hypothesis is that HIF-1 levels and thus HIF-1 signaling in the developing myocardium will be reduced by TCDD treatment in vivo during a critical stage and in particularly sensitive sites for heart morphogenesis. This inadequate HIF signaling will in turn lead to the misexpression of many critical HIF-1 responsive genes resulting in outflow tract (OFT) and coronary vasculature defects. Our preliminary data was unexpected in that it showed a marked increase in HIF-1 protein levels by Western blot and a decrease in the intensity of immunostaining for HIF-1 alpha nuclear expression in the OFT myocardium of TCDD-treated embryos. This area at the base of the OFT is particularly hypoxic during normal development and where the right and left coronary arteries eventually originate. This result is consistent with TCDD interfering with the normal level of HIF-1 signaling during cardiac development that could in turn affect coronary vessel development. Supported in part by Grants HL65314, HL0775436, ES103507.

Platform Session V

19. Molecular and physiological mechanisms underlying embryonic cardiac rhythmicity in zebrafish

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Establishing a synchronized forceful contraction pattern of the heart is critical for the survival and growth of vertebrate embryos. However, while molecular components essential for cardiac patterning have been identified in recent years, mechanisms underlying the initiation and maintenance of rhythmic cardiac contraction remain to be explored. From the large-scale zebrafish genetic screens, we identified multiple alleles of tremblor (tre) mutants. Homozygous tre mutant embryos develop a dysmorphic and fibrillating heart, indicating the essential role of tre in embryonic cardiac morphogenesis and rhythmicity. By positional cloning, we showed that tre locus encodes a cardiac-specific Sodium Calcium Exchanger gene, NCX1h, in zebrafish. High-speed confocal imaging revealed calcium transients defects in tre cardiomyocytes. The calcium waves that normally accompany cardiac contractions were absent in tre, rather the sporadic contractions were accompanied by local, unsynchronized fluorescence signals. These data demonstrate a causative relationship between calcium flux and cardiac fibrillation and indicated that tre mutants can serve as a model for abnormal calcium homeostasis induced cardiac fibrillation. To further dissect the genetic networks critical for cardiac rhythmicity, we screened a diversity oriented chemical library for suppressors of the cardiac fibrillation phenotype in tremblor mutant embryos. From this screen, we identified a compound that suppresses cardiac fibrillation, restores synchronized rhythmic cardiac contraction and maintains normal cardiac morphology in tre mutant embryos. Cellular mechanisms by which this compound suppresses the fibrillation phenotype in tre and the physiological impacts of this compound on wild type zebrafish embryonic hearts and isolated mammalian cardiomyocytes will be discussed.

20. Tbx3 acts as a genetic switch for heart pacemaker formation

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The sinoatrial node (SAN) is the dominant pacemaker of the heart responsible for generation of the heartbeat. The SAN has a distinct phenotype and gene expression program compared to the bordering atrial cells, which is essential for pacemaker function. Despite the crucial role of the SAN in heart function, the mechanisms that underlie its specification and formation are not known. Tbx3 is a transcriptional repressor that is expressed in all developing and mature central conduction system components of the heart, including the SAN. Genetic lineage analysis showed that after the atrial gene program has been initiated, atrial cells do not contribute to SAN formation, suggesting that the SAN develops from an early specified Tbx3-positive precursor population. We generated Tbx3-deficient mice to assess the function of Tbx3 in the SAN region and found that Tbx3-deficiency leads to expansion of atrial gene expression in the SAN domain and a partial loss of the SAN gene program. Next, we expanded the Tbx3 expression domain into the atrial myocardium by using mice that conditionally activate Tbx3. In atria of these mice expression of functionally important atrial genes, including Cx40, Cx43 and Scn5a, was down-regulated, whereas expression of genes important for pacemaker function, such as Hcn4, was induced. Furthermore, these mice had arrhythmias and developed functional ectopic nodes in the atria, thus demonstrating the ability of Tbx3 to convert atrial cells into functional nodal cells in vivo. Together, these data identify a Tbx3-dependant pathway for specification and formation of the SAN and show that Tbx3 acts as a genetic switch for pacemaker phenotype and function.

21. Genetic analysis of Popdc1 and Popdc2 function in mouse and zebrafish heart

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The Popeye domain containing (Popdc) gene family encodes membrane proteins with a preferential expression in striated muscle. In order to analyze their function we have created Popdc1 and Popdc2 null mutant mice. Both mouse strains displayed normal viability during postnatal life. Detailed analysis of the LacZ expression pattern of both mutants revealed a strong expression in the cardiac conduction system (CCS) of adult mice, which was higher than in working myocardium. Electrophysiological analysis of both mutants revealed cardiac conduction abnormalities. Popdc1 mice displayed a sinus bradycardia and a retarded AV node conductivity, which became pronounced after beta-adrenergic stimulation. Popdc2 developed a load-induced sinus bradycardia after subjecting mutant mice to an exercise test or to prolonged mental stress by airjet. The stress-induced bradycardia in Popdc2 mice was due to a sinus bradycardia and not due to prolonged AV conduction or AV nodal block. Histological analysis revealed structural defects in the sinus node morphology in case of Popdc2 null mutants. Using a morpholino-based knock-down of popdc2 in zebrafish embryos revealed an essential function of popdc2 for cardiac and skeletal muscle development in zebrafish. Injected embryos displayed a disorganized tail musculature and a dysmorphic heart. At low morpholino concentrations some of the morphant's hearts displayed cardiac arrhythmia, which was reminiscent of the conduction abnormalities observed in the adult mouse heart. In conclusion the Popdc family plays an important role for heart and muscle development in zebrafish and appears to be essential for the conduction system of the adult mouse heart. Supported by DFG, BR1218/10-4.

22. Clonal analysis of the origin of the mammalian ventricular conduction system

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The cardiac conduction system is composed of specialised cardiomyocytes which play a critical role in the generation and propagation of electrical activity through the heart to coordinate cardiac contraction. A retrospective clonal analysis was performed in order to establish the lineage relationship between cells of the ventricular conduction system and the surrounding working myocardium. The a-cardiac actinnlaacZ clonal analysis mouse line was crossed with the Cx40-eGFP transgenic line in which cells of the conduction system are easily detectable. Only 0.1% of β-galactosidase positive clusters at 3 weeks after birth were found to colocalise with eGFP positive cells in accordance with estimates of the relative number of conductive cardiomyocytes in the heart. Two types of cluster can be distinguished: small clusters (less than 40 β-galactosidase positive cells) and large clusters. Immunohistochemistry performed on sections showed that small clusters are composed of cells of either conductive or contractile myocardium but not both. In contrast, large clusters are formed by a mixed population of conductive (Cx40-eGFP positive) and contractile myocytes. None of the observed clusters encompassing elements from the central conduction system, i.e. His bundle, include elements from the peripheral conduction system, i.e Purkinje fibers. Our results suggest that in the mammalian heart conductive and contractile cardiomyocytes share a common progenitor and that the central and the peripheral conduction system develop independently. Furthermore, the existence of small clusters exclusively in contractile or conductive myocytes suggests that the differentiation of common progenitor cells into conductive or contractile pathways occurs during a restricted time window during development, and that thereafter conductive or the contractile myocytes proliferate by outgrowth.

23. Deletion of the cardiac L-type calcium channel (CaV1.2) causes embryonic death

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Background: Cardiac function depends upon calcium transients which are initiated by the sarcolemmal L-type calcium channel (LTCC). These cyclic changes in calcium levels also appear to regulate gene expression in the developing and mature heart. In addition, we have previously shown that blockade of the LTCC in cultured embryos leads to abnormalities of the cardiac outflow tract, which is derived from the Anterior Heart Field (AHF). Objective: To determine whether global and AHF-specific LTCC expression is required for normal cardiac morphogenesis and embryonic survival. Design/Methods: Global deletion of the major cardiac LTCC isoform, CaV1.2, was obtained using floxed-CaV1.2 mice (LoxP sites flanking exons 14 and 15) mated to β actin-Cre mice. Conditional deletion of CaV1.2 in the AHF was obtained using Mef2c-AHF-Cre mice rovided by Dr. Brian Black. Mice were harvested at various stages of gestation and after birth and analyzed for genotype and survival. Specimens were also examined for changes in gross morphology and cardiac morphology by visual observation and histological methods. Results: Full term litters from pairings to create both global and conditional deletion of CaV1.2 contain no knockout mice and about 33% wild type (+/+) and 66% heterozygote mice, indicating that the expression of CaV1.2 throughout the embryo and specifically in the AHF and OFT is required for survival to term. Mice with global deletion of CaV1.2 survive until embryonic day (E) 12-13. Litters harvested at E13.5 contain the normal Mendelian ratio of the three possible genotypes, but -/- embryos are friable, do not have beating hearts, and have the gross morphology of E11.5-12 embryos, suggesting that they have arrested development, and/or death, at this age. Hearts of E13.5 -/- mice also appear to have a 1-2 day delay in development, but appear structurally normal by gross observation. Embryos harvested at earlier stages of development are viable, appear to have normal development, and contain beating hearts which are grossly normal. Further experiments are ongoing to examine the cause of embryonic demise and the effects of CaV1.2 deletion on cardiac structure, function, and gene expression. Conclusions: Normal expression of the major cardiac LTCC isoform is required for survival past the mid-

embryonic period. Embryos lacking CaV1.2 die around E12 with grossly normal hearts and the reason for embryonic death is currently being investigated.

Platform Session VI

24. Endocardial Brg1 Represses ADAMTS1 to Maintain the Microenvironment for Myocardial Morphogenesis

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Myocardial trabeculation initiates when clusters of ventricular myocardial cells respond to endocardial signals and protrude into the cardiac jelly separating the myocardium from the endocardium. Endocardial cells invaginate between the elongating myocardial projections, becoming tightly associated with the myocardial cells as the cardiac jelly dissipates. Later, the trabeculae largely collapse to thicken the compact layer of the myocardium. Excessive trabeculation or failure of the trabeculae to undergo compaction causes cardiomyopathy in humans. We show that Brg1, the core component of the BAF complex, a SWI/SNF-like ATP-dependent chromatin remodeling complex, is required in the endocardium for myocardial trabeculation. Surprisingly, the expression of multiple genes known to regulate trabeculation remains normal in the absence of endocardial Brg1. Instead, the trabeculation defects are caused by derepression of a secreted matrix metalloprotease, ADAMTS1, which is normally not expressed at high levels in ventricular endocardium until later in development. Derepression of ADAMTS1 causes premature breakdown of the cardiac jelly and termination of trabeculation, suggesting that cardiac jelly regulation by endocardial cells coordinates maturation of the ventricles. We further show that ADAMTS1 is a direct transcriptional target of the BAF complex in endothelial cells. Our studies demonstrate that transcriptional repression of a secreted matrix protease within one tissue layer, the endocardium, provides an appropriate environment for the neighboring myocardial tissue to respond to developmental cues. This observation supports a general theme where chromatin regulation influences tissue morphogenesis by regulating the microenvironment.

25. Myocardial Smad4 Is Essential for Cardiogenesis in Mouse Embryos

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Accumulated evidence indicates that TGFβ/BMP signaling pathways play critical roles during cardiogenesis. Smad4 encodes the only common Smad protein in mammals, which is a critical nuclear mediator of TGFβ/Bmp signaling. The aim of this work was to investigate the roles of Smad4 during heart development. To overcome the early embryonic lethality of Smad4-/- mice, we specifically disrupted Smad4 in the myocardium using a Cre/loxP system. We show that myocardial-specific inactivation of Smad4 caused heart failure and embryonic lethality at mid-gestation. Histological analysis revealed that mutant mice displayed a hypocellular myocardial wall defect, which is likely the primary cause for heart failure. Both decreased cell proliferation and increased apoptosis contributed to the myocardial wall defect in mutant mice. Data presented in this presentation contradict a previous report showing that Smad4 is dispensable for heart development. Our further molecular characterization indicated that expression of Nmyc and its downstream targets, including cyclin D1, 2 and Id2, were downregulated in mutant embryos. Reporter analysis indicated that the transcriptional activity of the 351bp Nmyc promoter can be positively regulated by BMP stimulation and negatively regulated by TGFβ stimulation. Chromatin immunoprecipitation (ChIP) analysis revealed that the Nmyc promoter can form a complex with Smad4, suggesting that Nmyc is a direct downstream target of Smad4. In conclusion, this study provides the first mouse model showing that Smad4 plays essential roles during cardiogenesis.

26. Sonic hedgehog Modulates Addition of the Secondary Heart Field to the Arterial Pole
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The arterial pole of the heart is formed by the secondary heart field (SHF). Myocardial cells are added first to the truncus, and smooth muscle later surrounds the aortic sac. Ablation of the secondary heart field causes malalignment of the aorta and pulmonary trunk, resulting in conotruncal malformations such as double outlet right ventricle and Tetralogy of Fallot with pulmonary stenosis or atresia. This study investigates signaling pathways that may be responsible for the addition of myocardium from the SHF to the arterial pole. Because Sonic hedgehog (Shh) is a known proliferative factor and is located in the pharyngeal endoderm underlying the SHF, its contribution to normal arterial pole development is likely critical. Myocardium adds to the arterial pole from the SHF between stages 14 and 18 in the chick, so these stages were targeted for analysis. While Shh mRNA is produced in the pharyngeal endoderm adjacent to the SHF, Shh protein is found within the SHF along with its receptor, patched2, and downstream target Gli3. Patched2 exhibits a gradient, with low expression levels in cranial SHF and higher expression in more caudal SHF. This supports a morphogen effect for Shh on the SHF. In vitro, blocking the Shh pathway in SHF explants reduces migration and proliferation. In ovo, blocking Shh at the beginning of the myocardial contribution also reduces migration, inhibits proliferation, and affects differentiation. If these embryos are developed further, mature hearts exhibit persistent truncus arteriosus, mis-patterning of the aortic vessels, pulmonary stenosis, and ventricular septal defects. However, the cardiac neural crest, which is responsible for patterning the pharyngeal arch arteries and providing the outflow tract septum, migrates correctly. These experiments indicate an essential role for Shh in the normal addition of myocardium from the SHF to the developing arterial pole. Supported by PHS Grants HL083240 and HL070140.

27. BMP4 Function is Required in Second Heart Field-Derived Myocardium for Endocardial Cushion Remodeling, Outflow Tract Septation, and Semilunar Valve Development
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The most severe forms of congenital heart disease are those that involve defects in valve development and septation of the common ventricular chamber and outflow tract. Recently, these structures have been shown to arise from a novel cardiac progenitor population referred to as the second heart field (SHF). The SHF is a population of embryonic mesoderm that is responsible for the formation of the right ventricle, ventricular septum and outflow tract as well as the aortic and pulmonic valves. Despite the prevalence of defects in these SHF-derived structures, critical details involved in the molecular regulation that controls valve and septum formation remain to be determined. One group of signaling molecules that are critical to heart development includes the bone morphogenetic proteins (BMPs). Mice that lack BMP4 die early in development, before heart formation begins. Therefore, we have employed a conditional approach to inactivate BMP4 exclusively in the SHF in order to define the requirement of BMP4 for outflow tract cushion development. Conditional inactivation of BMP4 in the SHF results in mice that are born with persistent truncus arteriosus and have abnormally formed semilunar valves. Detailed examination of these animals shows that the cardiac jelly forms at the appropriate time and that endocardial to mesenchymal transition occurs normally. Thus, initial formation of the endocardial cushions is normal, however the cushions fail to undergo normal expansion and remodeling and as a result the common outflow tract does not fully septate and the semilunar valves do not form normally. These results provide critical new information about the processes that underlie normal heart development and how disruption of these events leads to developmental anomalies resembling congenital heart defects in humans.

28. Retinoic Acid Deficiency Alters Secondary Heart Field Formation and NKx2.5 Regulation
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Retinoic acid (RA), the active derivative of vitamin A, has been implicated in various steps of cardiovascular development. The retinaldehyde dehydrogenase 2 (RALDH2) enzyme catalyzes the second oxidative step in RA biosynthesis and its loss of function creates a severe embryonic RA deficiency. Raldh2-/- knockout embryos fail to undergo heart looping and have impaired atrial and sinus venosus development. To understand the mechanism(s) producing these changes, we have examined the contribution of secondary heart field (SHF) to pharyngeal mesoderm, atria and outflow tract in Raldh2-/- embryos. RA deficiency alters SHF expression in two separable ways. First, Raldh2-/- embryos exhibit an expansion in the posterior limit of endogenous markers of the SHF including Tbx1, Isl1, Fgf8, and of Mlc1v-24–lacZ reporter transgene expression, indicating that endogenous RA is required to restrict the SHF posteriorly. Second, RA appears to upregulate cardiac Bmp6, Bmp7 and Fgf8 levels at the looping stage. Utilization of different transgenes suggests that contribution of the SHF to both inflow and outflow poles is perturbed under RA deficiency, creating a disorganization of their derivatives. We are currently examining whether defects in growth factor expression are responsible for these recruitment defects. We have also investigated genetic crosstalk between Nkx2.5 and RA signaling in the primary heart field by generating double mutant mice. Nkx2.5 haploinsufficiency or complete loss of function increases sinoatrial formation and rescues Tbx5 expression in Raldh2-/- mutants. The regulation of Nkx2.5 by RA signaling may be direct, as the upstream regulatory region of the Nkx2.5 gene contains five evolutionarily conserved RA-response elements of the 'direct-repeat 5' (DR5) type. A BAC transgene encompassing the Nkx2.5 RA-response elements drives lacZ reporter expression in the cardiac crescent. While the early domain of expression of this transgene is not altered at the crescent stage in Raldh2-/- mutants, it is then reduced in the outflow tract and the right ventricle of mutants at E10.5. RA deficiency also reduces Fgf signaling and Fgf10–lacZ transgene expression at this stage, indicating that RA is necessary for the proper function of the SHF in controlling outflow tract septation.

Platform Session VII

29. Distinct Functions of the MAP3Kinases, MEKK3 and MEKK4, for Heart Valve Development
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Congenital heart defects (CHDs) occur in one percent of all births. While gene mutations have been identified in CHD patients, not much is known about coordinated signal transduction mechanisms during heart morphogenesis. The endocardial cushions of the atrioventricular canal and outflow tract contribute to the formation of valves and septa in the heart. Epithelial cell to mesenchymal cell transition (EMT) is a key process in cardiac cushions before this tissue undergoes remodeling into valves and septa. Defining the complex signaling networks directing cardiac cushion epithelial to mesenchymal transition (EMT) is essential for understanding the etiology of congenital heart defects. We have identified the MAP3Kinases, MEKK3 and MEKK4, as signaling components present during cardiovascular development. MEKK3 and MEKK4 message is detected to the developing mouse heart. Furthermore, MEKK3 protein is detected in myocardium and endocardium surrounding the cardiac cushions of the atrioventricular canal during heart morphogenesis, while MEKK4 is found in the myocardium, endocardium, and cushion mesenchyme. Functional assays were employed to examine how MEKK3 and MEKK4 kinase activity contributes to cardiac cushion EMT. Addition of a dominant negative (dn) MEKK3 or dn-MEKK4 to endocardial cushion explants, cultures that recapitulates in vivo EMT, causes a significant decrease in mesenchyme

formation as compared to controls. Additionally, ventricular explant cultures, where the endocardial cell lining does not normally undergo EMT, provided with constitutively active (ca) MEKK3 activates mesenchyme production, however, ca-MEKK4 is not sufficient to cause EMT in this system. Furthermore, ca-MEKK3 expression in ventricular explants causes an increase in secreted TGF beta2, which is a known mediator of mesenchyme formation. Our observations have defined MEKK3 and MEKK4 expression during cardiovascular development and suggest that MEKK3 and MEKK4 have diverse functions during development of heart valves and septa. These important findings may contribute to the development of tissue engineering strategies or therapies for people with CHDs.

30. Tbx20 and Twist1 function in endocardial cushion mesenchyme

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In the US, congenital heart defects (CHD) are among the most common birth defects, occurring in approximately 1/100 live births. The majority of CHDs involve abnormal valvuloseptal development. Heart valvulogenesis is initiated by the formation of proliferative, undifferentiated, endocardial cushions, which will ultimately remodel into mature valve leaflets. While much is known about early events in valve development, the molecular mechanisms underlying the transition from endocardial cushion to remodeling valve leaflet remain relatively unknown. Endocardial cushions are composed of highly proliferative, migratory, undifferentiated, mesenchymal valve progenitor cells embedded in an unorganized extracellular matrix (ECM). We have previously shown that robust expression of Tbx20, a T-box transcription factor, in response to BMP2 signaling regulates proliferation and mesenchymal gene expression in endocardial cushions. In addition, new evidence suggests that Tbx20 promotes endocardial cushion cell migration. Little is currently known about other factors that function with Tbx20 to coordinate endocardial cushion maturation. The basic helix-loop-helix transcription factor Twist1 is expressed in a variety of mesenchymal structures and has been implicated in maintaining proliferative, undifferentiated, migratory populations of cells. Here we report that Twist1 is expressed in developing endocardial cushions and is responsive to BMP2 signaling. In addition, loss of Twist1 function via Twist1-specific siRNA results in decreased Tbx20 expression. Taken together, these findings provide evidence that Twist1 acts upstream of Tbx20 in order to maintain a proliferative, undifferentiated, migratory mesenchymal cell population in endocardial cushions. This work was supported by AHA Ohio Valley Affiliate pre-doctoral fellowship 0515153B.

31. TGF-beta2 is required in vivo for epithelial-mesenchymal transformation, collagen fibrollogenesis, and differentiation, condensation and maturation of mesenchyme during valvulogenesis

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Cardiac valve thickening is a major cardiovascular manifestation of syndromic and non-syndromic valvular diseases. Mutations in TGFBR1 or TGFBR2, and a SMAD2-interacting protein, flamin A (FLNA) are found in Marfan syndrome (MFS)/Ehlers-Danlos syndrome (EDS), and X-linked myxomatous valvular dystrophy (XMVD), respectively. Although biochemical analyses of these mutations in cell culture suggest a loss of TGF-beta signaling, analyses of clinical specimen of MFS demonstrate a paradoxical increase in TGF-beta ligands and TGF-beta signaling. The role of TGF-beta ligands in valvulogenesis remains unclear. We hypothesize that TGF-beta2 plays essential role in vivo in valvulogenesis. Here, we demonstrate that TGF-beta2-deficient mice (~33%) develop abnormal thickening and myxomatous changes in cardiac valves. Dysregulated epithelial-mesenchymal transformation (EMT), enhanced cell proliferation, and reduced apoptosis, differentiation, maturation and

condensation of valvular mesenchyme significantly contribute to the myxomatous valvulogenesis in these mice. In addition, expression of lumican, collagen, elastin, lysyl oxidase and periostin, all of which are required for collagen fibrollogeneis, is significantly reduced in cardiac valves of TGF-beta2-deficient mice. Consistently, we find that adult lumican-deficient mice develop valve thickening. Finally, we show that the cardiac valve thickening in TGF-beta2-deficient mice is accompanied by a significant reduction in 'canonical' TGF-beta signaling. Collectively, these data demonstrate an essential developmental function of TGF-beta2 in vivo in EMT, collagen fibrollogenesis, and cardiac valve remodeling. The data also suggest that components of collagen fibers, including lumican are potential in vivo targets of TGF-beta2 signaling during valvulogenesis. Importantly, the valvular thickening in TGF-beta2-deficient mice has striking resemblance to the collagen-deficient valves of EDS and XMVD, and not of MFS that is accompanied by excessive collagen-matrix and TGF-beta signaling. These findings have significant implications since the NHLBI is currently conducting a clinical trial using Losartan, a drug that non-specifically inhibit the production of TGF-beta ligands, to treat cardiovascular manifestations in MFS.

32. The Role of Cartilage Link Protein 1 (Crtl1) in Heart Development

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DNA microarray analysis of the mouse heart at 10.5-11.0 days of embryonic development, demonstrated the abundant expression of Cartilage Link Protein 1 (Crtl1) in the atrioventricular (AV) junction. Crtl1 is best known for its role in stabilizing the interaction between chondroitin sulfate proteoglycans (CSPGs) and hyaluronan (HA) in the extracellular matrix (ECM). Immunohistochemical studies revealed that, during cardiac development, Crtl1 is initially expressed in the endocardium and cushion primordia. In these tissues, it is co-expressed with its putative cardiac binding partners HA and CSPG2 (versican). As development progresses, Crtl1 expression becomes restricted to the ECM of the endocardial cushions in the AV junction and outflow tract (OFT). Analysis of the Crtl1 knockout mouse shows a spectrum of cardiac malformations that affect the AV mesenchymal-derived tissues and abnormalities involving the myocardium of the ventricular wall and septum. Defects related to the malformation of the AV junctional tissues include AVSDs, DORV, and common AV canal. Abnormalities associated with the myocardial structures include "thin ventricular myocardium", and muscular VSDs. These myocardial abnormalities were unexpected given that Crtl1 expression, in the heart, is limited to the endocardium and endocardial-derived tissues. As we determined that, when compared to wild type littermates, Crtl1-/- embryos have significantly reduced levels of versican expression, we hypothesized that the observed malformations in the Crtl1 knockout mouse could be versican mediated. Thus, we expanded our studies to the Hdf (insertional versican mutant) mouse. Immunofluorescent labeling showed reduced levels of versican in the AV cushions of heterozygote Hdf mice and subsequent histological analysis of these embryos revealed cardiac defects similar to those seen in Crtl1-/- embryos. While the mechanisms via which the Crtl1/versican/HA complex is involved in heart development remain to be elucidated, the data presented clearly demonstrate that Crtl1 plays a significant role in valvuloseptal morphogenesis.

33. Endocardial cells transformation is dependent on Par6 regulation of RhoA

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Transforming Growth Factor β (TGFβ) regulates endocardial cell epithelial mesenchymal transformation (EMT) and remodeling during valvulogenesis. Data from our laboratory has suggested that activation of the Type I TGFβ receptor isoform ALK2 is sufficient and required for EMT, whereas ALK5 activation is not sufficient. To determine if ALK5 kinase activity is required for EMT, we used two structurally dissimilar ALK5 kinase inhibitors. ALK5 kinase inhibition blocked atrioventricular cushion transformation (>70% inhibition) and TGFBR3-mediated gain-of-function in ventricle endothelial cells (>90%) suggesting ALK5 kinase activity is required for EMT in vitro. Overexpression in ventricular

endocardial cells of Smad1 or Smad3, downstream effectors of ALK1 and ALK5 respectively, did not alter EMT. Recent data has demonstrated a role for novel noncanonical (nonSmads) pathways in mediating EMT. One is an ALK5-dependent activation of Par6 followed by Par6 activation of Smurf1 and subsequent RhoA degradation. Here we demonstrate this pathway regulates endocardial cell EMT. Par6 overexpression leads to gain-of-function in normally nontransforming ventricular explants (>210% of control) and dominant negative (dn) Par6 overexpression leads to loss-of-function (reduced EMT, >35%) in normally transformation competent atrioventricular cushion explants. Therefore Par6 is necessary and sufficient for endocardial cell EMT in vitro. Par6 activation has been shown to recruit the E3 ubiquitin ligase, Smurf1, leading to RhoA degradation and EMT. Overexpression of Smurf1 or dnRhoA lead to gain-of-function in the ventricle (>300%; >220%), while constitutively active (ca) RhoA blocks EMT (>90%). These data are consistent with a role for Smurf1-mediated loss of RhoA activity mediating transformation in vitro. Neither Rac1 or Cdc42 are a substrate for Smurf1 and overexpression of ca and dn constructs does not support a role for either in EMT. These data together suggests that a novel noncanonical pathway mediated by ALK5, Par6, and Smurf1 ubiquination of RhoA can mediate EMT in vitro. Supported by HL52922.

Platform Session VIII

34. A new mutant series provides insights into sensitivity of heart development to Tbx1-mRNA dosage

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DiGeorge/del22q11 syndrome is caused by gene haploinsufficiency. Gene-targeting and mutational analyses identified Tbx1, a T-box transcription factor, as the major disease gene in humans and mice. Tbx1+/- mice have a very mild phenotype, while Tbx1-/- mice have a very severe one. In addition, while patients are characterized by remarkable phenotypic variability, mouse mutants presented very little variability. A reason for these differences may be that the mouse is less sensitive to Tbx1 mRNA dosage change than humans. To address this hypothesis, we have generated a series of 8 different genotypes at the Tbx1 locus, using combinations of a null and two different hypomorphic alleles. qRT-PCR analyses of embryo RNA revealed a nearly continuous variation of Tbx1 mRNA. We found that the thymic phenotype followed almost linearly the dosage variation, whereas neonatal lethality followed a biphasic curve. Interestingly, 100% lethality occurred at a dosage reduction insufficient to cause cardiac defects, indicating that these are not the only lethal defects in Tbx1 mutants. Cardiac outflow tract (OFT) abnormalities occurred in a range of dosage between 22% and 14%. Within this range, the OFT phenotype was sensitive to very small dosage changes. Morphological analysis suggested that septation defects might be due to growth failure of the aortic portion of OFT. Interestingly, at this dosage level, we observed a broad OFT phenotypic variability, suggesting that variability is a function of dosage. Presumably, when dosage approaches a threshold level, stochastic events play a relatively more important role in determining phenotypic outcome. Thus, a higher sensitivity to dosage in humans may be the cause not only of more severe phenotype but also of higher variability in patients compared to heterozygous mice. Our mutant series provide a new tool to address mechanisms of gene dosage sensitivity and for determining transcriptome response to Tbx1 dosage in vivo.

35. Titration of Pax3 expression levels during morphogenesis of the outflow tract

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Pax3 is a transcription factor that is expressed in the neural tube, neural crest and somites during early embryogenesis. Homozygous loss-of-function mutations (such as in Splotch2H/Pax3-deficent mice) results in a wide spectrum of congenital defects including the cardiovascular defects: persistent truncus arteriosus (PTA), interventricular septal defects (VSD) and abnormal pharyngeal arch artery remodeling

anomalies. These cardiovascular defects are thought to result from a significantly reduced number of migratory cardiac neural crest cells. While it is well established that loss (referred to 0%) of functional Pax3 protein can lead to the aforementioned heart defects, the actual threshold level of functional Pax3 required for normal heart development to occur is presently unknown. Via use of compound Pax3 null and hypomorphic alleles (which respectively encode 0% and 10% functional Pax3 protein relative to that of the wildtype allele), we are able to generate mice which express 60%, 50%, 20% and 10% levels of functional Pax3 protein. Characterization of these mice has established a hierarchy of cell lineage sensitivity to reduced Pax3 levels, including those involved in outflow tract morphogenesis. These observations raise an intriguing question as to whether this lineage-restricted differential sensitivity to reduced Pax3 expression is due to the presence of redundant or compensatory mechanisms that are set at different levels in different cell lineages.

36. Polycomb Repressive Complex I plays a cell autonomous role in cardiac development

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Cell specialization plays a key role in cardiac development, and is created by heritable changes in gene expression that are maintained in part by chromatin modifications such as ubiquitylation, methylation and acetylation. The Polycomb Repressive Complex I (PRC1) contains the histone ubiquityl ligase Ring1b and functions to silence gene expression. Mel18 and Bmi1, members of PRC1, are necessary for antero-posterior patterning, regulation of cell proliferation, and stem cell maintenance. Here we show that Mel18 and Bmi1 are necessary in a dose dependent manner for heart, thymus and palate development. Mel18-/-;Bmi1+/- and Mel18+/-;Bmi1-/- embryos have cardiac malformations (atrioventricular septal defects, double outlet right ventricle, common arterial trunk and aberrant aortic arches). Conditional deletion of Ring1b was used to explore the requirement of PRC1 in cardiac development. Deletion in the cranial and cardiogenic mesoderm using Mesp1Cre results in embryonic lethality shortly after 10.5 dpc, with cardiac looping defects and pericardial oedema at 100% penetrance. Deletion in the cardiogenic mesoderm using Nkx2.5Cre results in atrioventricular septal defects (89%) and double outlet right ventricle (22%) but not aortic arch defects. Deletion in the cardiogenic and lateral plate mesoderm and endoderm using Lefty2Cre results in abnormal cardiac positioning to the left (57%), atrioventricular septal (86%), outflow tract (29%) and aortic arch defects (57%). Conditional deletion in the neural crest using Wnt1Cre does not result in cardiac, outflow tract or aortic arch defects but in craniofacial malformations (100%). These results indicate that Ring1b is required in a cell autonomous manner in the mesoderm during cardiac development. Although Ring1b is required in a cell autonomous manner in the neural crest this function is not necessary for cardiac development. We suggest that histone ubiquitylation plays a key role in cardiogenic mesodermal cell specialization.

37. Multiple functions of EIIIA and EIIIB splice isoforms of fibronectin in cardiovascular development

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Fibronectin (FN) is a multidomain, alternatively spliced, extracellular matrix protein essential for cardiovascular development. In the absence of FN, embryos die by e9.5 from severe cardiovascular defects. Alternatively spliced variants of FN containing EIIIA and EIIIB exons are expressed around embryonic vasculature and in cardiac cushions of the heart, and are evolutionarily conserved across species in their sequence and expression pattern. However, the function of these splice variants have remained a mystery since their discovery twenty years ago. Expression analysis suggested that EIIIA and EIIIB might function in cardiovascular development and/or disease, since they are expressed around developing blood vessels in physiological and pathological conditions but are downregulated around quiescent vasculature. EIIIA or EIIIB single-knockout animals are viable and fertile, and our analysis of pathological and physiological angiogenesis in these mice did not reveal their functions. To uncover the

functions of EIIIA and EIIIB, we deleted both EIIIA and EIIIB exons from the mouse genome. Interestingly, majority of the double null embryos (about 80%) die by e10.5 from cardiovascular defects. These defects include aberrant placental vascularization, defective remodeling of embryonic head and yolk sac vasculature, and heart defects. In addition, we discovered that EIIIA and EIIIB spliced variants are highly enriched around embryonic dorsal aortae compared with anterior cardinal veins, and we found a statistically significant decrease in the numbers αSMA-positive cells around the aortae at e9.5 as well as a delay in recruitment of these cells toward the aortae by e10.5. In addition, some of the double-null embryos developed defects in cardiac cushion formation such as multilayering of endothelial cells in the outflow tract. Taken together, our data indicate the role for EIIIA and EIIIB splice variants of FN in differentiation and/or recruitment of αSMA-positive cells, as well as in vessel remodeling, placental angiogenesis and cardiac cushion development.

38. Loss of fibulin-1 in mice causes a DiGeorge syndrome-like phenotype

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Fibulin-1 is an extracellular matrix protein implicated in cardiac development owing to its expression in the endocardial cushions of the developing heart. Here we report that mouse embryos homozygous for a fibulin-1 gene trap insertion are deficient in fibulin-1 and exhibit cardiac septation defects, ventricular wall thinning and abnormalities of outflow tract (OFT) remodeling that result in double outlet right ventricle and overriding aorta. Furthermore, fibulin-1 nulls also display anomalies of aortic arch arteries, hypoplasia of the thymus and thyroid as well as reduced size and under mineralization of bones of the skull. The spectrum of malformations is consistent with fibulin-1 playing a role in neural crest cell involvement in the development of the OFT, aortic arch arteries, pharyngeal glands and cephalic skeleton. Fibulin-1 in situ hybridization analysis shows that fibulin-1 is expressed by populations of neural crest cells that contribute to the pharyngeal arches and OFT. Furthermore, fibulin-1-deficient embryos display alterations in the numbers and patterning of neural crest cells expressing Crabp1 and Sox-10. Studies are ongoing to define the role of fibulin-1 in the differentiation, survival and/or guidance of neural crest cells. Based on the available evidence there is sufficient similarity between the phenotype of the fibulin-1-deficient embryos and humans with the neural crest-related disorder, DiGeorge syndrome (DGS), to suggest that fibulin-1 is a regulator of the pathway that is dysfunctional in DGS.

Platform Session IX

39. A Novel Murine Model with Features of Vacterl and Caudal Regression Indicates a Common Genetic Origin for both Syndromes

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The VACTERL and Caudal Regression Syndromes (CRS) are associated with cardiac malformations. VACTERL syndrome (OMIM 192350) is characterised by Vertebral, Ano-rectal, Cardiac, Tracheo-esophageal fistula, Esophageal atresia, Renal and Limb malformations. CRS (OMIM 600145) is associated with skeletal malformations (sacral agenesis), presacral mass, anorectal and genitourinary malformations. In a phenotype driven recessive ENU mutagenesis screen for cardiac malformations, using high-throughput magnetic resonance imaging as a screening tool, we have identified a mouse line (RECC19) with a phenotype resembling an overlap between VACTERL and CRS. These include: cardiac malformations (VSD, DORV, abnormal aortic arch), presacral mass, ano-rectal malformations, axial skeletal defects (absent tail, sacral agenesis, increased number of thoracic vertebrae), hypoplastic hind

limbs, renal agenesis, and abnormal tracheo-oesophageal septation. It shows a recessive inheritance pattern with 100% penetrance. Using a genome-wide screen we have mapped the mutation causing this phenotype to a 2Mb region on Chr19. Sequencing all candidate genes in this region reveals only one with a non-synonymous mutation. The functional effects of this mutation are being characterized. Our data indicates that a hitherto unsuspected common genetic origin can exist for both VACTERL and CRS.

40. Mediating ERK1/2 Signaling Rescues Congenital Heart Defects in a Mouse Model of Noonan Syndrome

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Noonan syndrome (NS) is an autosomal dominant disorder characterized by a spectrum of defects, including short stature, craniofacial anomalies and congenital heart disease (CHD). NS is the most common nonchromosomal cause of CHD and 80-90% of NS patients have cardiac involvement. Multiple mutations within the protein tyrosine phosphatase SHP2 are responsible for approximately 50% of the cases of NS with cardiac involvement. For at least some of the mutations, the mutant protein exhibits increased phosphatase activity, resulting in a gain-of-function. To understand the developmental stage- and cardiomyocyte-specific consequences of the NS SHP2 gain-of-function mutation, the β- and α-myosin heavy chain (MHC) promoters were used to express Q79R in the fetal and postnatal ventricles, respectively. β-MHC-Q79R hearts showed selective ERK1/2 activation throughout the latter half of gestation, leading to altered cardiomyocyte cell cycling, ventricular noncompaction and ventricular septal defects (VSDs) with 39% of the mice dying of congestive heart failure by 8 months. In contrast, despite chronic ERK1/2 activation in the post-birth cardiomyocytes, α-MHC-driven expression of Q79R was completely benign. To test the hypothesis that down-regulating cardiomyocyte ERK1/2 hyperphosphorylation during embryogenesis would restore normal cardiac architecture and function, the β-MHC Q79R mice were crossed into erk1 and erk2 knockout mice. These mice both showed reduction of inappropriate ERK1/2 hyperphosphorylation throughout embryogenesis, resulting in normal cardiac cell cycling, anatomy, function and improved survival rate, confirming the pathway's necessity and sufficiency in mediating mutant SHP2's effects. Our data establish the developmental stage-specific effects of Q79R cardiac expression in NS, show that ablation of subsequent ERK1/2 activation prevents the development of cardiac abnormalities and suggest that ERK1/2 modulation could have important implications for developing therapeutic strategies in CHD.

41. Muscleblind 2, a RNA binding protein important in Myotonic Dystrophy and Cardiac Conduction

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The Muscleblind genes (Mbnl1, Mbnl2, and Mbnl3) contain conserved tandem zinc finger domains, and they are similar to the Drosophila muscleblind gene named for its importance in Drosophila muscle and eye development. Mbnl2 is critical for proper alternative RNA splicing, RNA localization and implicated in the pathogenesis of myotonic dystrophy (DM), a multisystem disorder characterized by pathogenic CTG/CCTG expansions. We generated Mbnl2 mice deficient that develop myotonia and muscular dystrophy (DM). Expression and mRNA splicing of the chloride channel (Clcn1) is defective, as well integrin a3 expression that is regulated at the level of RNA localization by Mbnl2. Atrial and ventricular arrhythmias are common in DM patients, and sudden cardiac death is responsible for up to 1/3 of DM1 deaths. The Mbnl2 mutant mouse has a cardiomyopathy with a dilated right ventricle. This is reminiscent of the a-actinin-associated LIM domain protein (Alp) deficient mouse that also has a right ventricle dilated cardiomyopathy. RT-PCR demonstrates that alternative RNA splicing of Alp is affected. We have further analyzed the function of Mbnl2 using rat primary cardiomyocyte monolayers. Overexpression of a dominant negative Mbnl2 mutant fused to RFP results in spontaneous reentry as determined by simultaneous optical voltage and calcium mapping. Connexin 43 protein and RNA

expression are also diminished with dominant negative Mbnl2 expression. Expression of wild type Mbnl2-RFP fusion, and RFP alone do not cause spontaneous reentry or alterations in connexin 43 expression. Our research demonstrates that Mbnl2 is essential for proper alternative pre-mRNA splicing and RNA cellular localization. Mbnl2 deficiency results in cardiac and skeletal muscle defects and contributes to the pathogenesis of myotonic dystrophy.

42. Duplication of the entire 22.9-Mb human chromosome 21 syntenic region on mouse chromosome 16 causes cardiovascular abnormalities

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Trisomy 21/Down syndrome is the most common genetic cause of congenital heart disease. The mouse is the most important model organism for Down syndrome since the regions on human chromosome 21 are syntenically conserved in three regions on mouse chromosomes 10, 16, and 17. Ts65Dn mice, the most widely used model for Down syndrome, are trisomic for approximately 56.5% of the human chromosome 21 syntenic region on mouse chromosome 16. Trisomy 16 mice are an important model for congenital heart defects in Down syndrome, but only approximately 23.3% of the trisomic region in trisomy 16 mice is syntenic to any region on human chromosome 21. The Tc1 trans-species mouse strain, carrying an almost entire human chromosome 21, exhibited variable levels of mosaicism of the extra chromosome in different tissues, confounding analysis of phenotypic consequences. To generate a more genuine mouse model for Down syndrome, we have established a 22.9-Mb duplication spanning the entire human chromosome 21 syntenic region on mouse chromosome 16 in mice using Cre/loxP-mediated long-range chromosome engineering. The presence of the intact duplication in mice was confirmed by fluorescent in situ hybridization and microarray-based comparative genomic hybridization. The expression levels of the genes within the duplication interval reflect gene dosage effects in the mutant mice. The cardiovascular abnormalities of the mouse model were similar to those of patients with Down syndrome. This new mouse model represents a powerful tool to further understand the molecular and cellular mechanisms of Down syndrome-associated congenital heart disease.

POSTER SESSION ABSTRACTS

43. Transgenic rescue of induced heart defects in Xenopus.
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Phage phiC31 encodes an integrase that can mediate the insertion of extrachromosomal DNA into genomic DNA. The phiC31 integrase system has been adapted to create transgenic cells and organisms in a variety of species. To use phiC31 integrase to generate transgenic Xenopus laevis embryos mRNA encoding integrase was co-injected with plasmid containing a GFP reporter into single cell embryos. Plasmids also contained insulator sequences to prevent position effects and the integrase attachment site attB. Southern blot analysis confirmed that a single copy of the plasmid was integrated into the genome. Tissue appropriate expression was achieved with the CMV promoter , a Xenopus 551 base-pair minimal gamma-crystalline lens promoter (expressed in lens) or a 10 kilobase-pair XNkx2-5 promoter (expressed in heart). Between 20 to 35% the embryos assayed one week after fertilization were transgenic. Expression levels of transgenic GFP driven by the XNkx2-5 promoter was equal to endogenous XNkx2-5 expression. Furthermore, if the XNkx2-5 promoter was used control expression of XNkx2-5 mRNA resistant to oligonucleotide inhibition, transgenic embryos that had lost endogenous XNkx2-5 expression via oligonucleotide inhibition were rescued. These experiments demonstrate that the integration of insulated gene sequences using PhiC31 integrase can be used to efficiently create transgenic embryos in Xenopus laevis and may increase the practical use of PhiC31 integrase in other systems as well.

44. Immortalized mouse epicardial cells serve as a model of epicardial differentiation in vitro
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TGFβ induces loss of epithelial character and smooth muscle differentiation in primary epicardial cells from chick and mouse. To facilitate the study of TGFβ signaling in epicardial cells we generated immortalized mouse cell lines. Epicardial cells were isolated from a transgenic mouse where the large T antigen is temperature regulated (Immortomouse, Jat et al., PNAS USA. 88:5096-5100, 1991). Cells can be maintained and expanded at 33ºC and at 37ºC large T antigen expression is silenced and cells revert to wildtype growth characteristics. Hearts from mouse embryos at E10.5, E11.5 and E13.5 were placed on collagen-coated slides for 12 h at 37ºC, the hearts removed, and the culture incubated at 33ºC. Cells grew as tightly packed epithelium and were expanded for storage in liquid nitrogen or replated. Cells lines have been in culture for over 6 months (E11.5) or at least two months (E 10.5 and E 13.5 lines). The E10.5 cell lines were isolated from Immortomouse X Sm22αLacZ matings. Both Sm22αLacZ positive and negative cell lines were established. Cells were replated and incubated at 37ºC for 24 h prior to the addition of 250 pM TGFβ1 or TGFβ2. Each induced the loss of epithelial morphology as monitored by the loss of Zonula Occludens-1 (ZO-1) at 72 h. TGFβ1 or TGFβ2 also induced the smooth muscle markers SM22α and calponin. The Sm22αLacZ cells up-regulate LacZ when they differentiate to smooth muscle. Inhibition of activin receptor-like kinase (ALK) 5 by 2.5 μM SB431542 blocked all effects of TGFβ1 or TGFβ2. Epicardial cells grown on matrigel form tube like structures and express the endothelial marker PECAM. These data demonstrate that immortalized epicardial cells are a suitable model for studying mechanisms of epicardial differentiation in vitro. Supported by HL52922, HL67105, AHA06551, HL076133, GM07347 & MOD Basil O'Connor 5-FY04-216.

45. Myocardium at the base of the aorta and pulmonary trunk is prefigured in the outflow tract and second heart field

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Outflow tract myocardium in the mouse heart is derived from pharyngeal mesodermal progenitor cells in the anterior part of the second heart field. We have recently characterized a transgene (y96-Myf5-nlacZ-16), which is expressed in a subdomain of the outflow tract and later predominantly in myocardium at the base of the pulmonary trunk. We now characterize the expression profile of another transgene, A17-Myf5-nlacZ-T55, which is expressed in a different population in the outflow tract and subsequently in myocardium at the base of the ascending aorta. At E9.5, expression of the two transgenes is detectable in progenitor cells located in different domains of the second heart field, suggesting that these cells contribute to different regions of myocardium in the outflow tract and subsequently to myocardium at the base of the great vessels. Analysis of the distribution of clones of cardiomyocytes in the arterial pole of the heart at E10.5 and E14.5, provides insight into the behaviour of myocardial progenitor cells of the outflow tract. At E14.5, clonally related clusters of cells are mainly observed either at the base of the pulmonary trunk or ascending aorta, supporting the conclusion that these are distinct myocardial compartments. At E10.5 large clones in subdomains of the outflow tract point to coherent growth, consistent with the presence of progenitor cells already organised in sub-domains of the second heart field. Our results suggest that myocardial differences at the base of the great arteries are prefigured in distinct progenitor cell populations within pharyngeal mesoderm, with important implications for understanding the etiology of congenital heart defects affecting the arterial pole of the heart.

46. Epiblastic Cited2 function explains pleiotropy and penetrance of cardiac malformation resulting from its deficiency

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Deletion of the transcription factor Cited2 results in highly pleiotropic and penetrant cardiovascular malformations and abnormal left-right patterning. We have used a conditional knockout approach to dissect the nature of cardiovascular pleiotropy arising from Cited2 deficiency. Deletion of Cited2 in the epiblast using Sox2Cre completely recapitulated the global knockout phenotype with high penetrance of cardiac malformations, adrenal agenesis, and laterality defects. Deletion of Cited2 in the neural crest using Wnt1Cre resulted in fused cranial ganglia but did not affect laterality, cardiac or adrenal development. Deletion in the cardiogenic mesoderm using Nkx2-5Cre, Mesp1Cre or Isl1Cre resulted in low penetrance of septal defect, but no outflow tract, aortic arch or laterality defects. Deletion in the mesoderm using TCre resulted in frequent adrenal agenesis, but infrequent cardiac septal and laterality defects. Quantitative RT-PCR showed that Cited2 expression was markedly reduced in Cited2-/flox; Nkx2.5Cre hearts in comparison to Cited2+/flox; Nkx2.5Cre hearts. In keeping with the absence of left-right patterning defect, and low frequency of septal defect, there was no difference in expression of the Cited2 target gene Pitx2c in Cited2-/flox; Nkx2.5Cre hearts. Using quantitative RT-PCR we also show that Pitx2c expression is markedly reduced in Cited2-/- hearts in comparison to Cited2+/+ hearts, but that there is substantial variation of Pitx2c expression in Cited2-/- hearts. These results indicate that the high penetrance and pleiotropy of cardiac malformation in Cited2 deficiency arises from an essential requirement in the epiblast, and not from a later cell-autonomous function in the mesoderm, and that phenotypic variation could arise from variation in Cited2 target gene expression. The inability to genetically dissociate pleiotropic cardiac malformation from laterality defect suggests that they are linked phenomena, and that abnormal left-right patterning may give rise to pleiotropic cardiac malformation.

47. Incorporation of Obscurin into Nascent Sarcomeres: Implication for Myofibril Assembly during Cardiac Myogenesis

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Obscurin is a recently identified giant multidomain muscle protein whose functions remain poorly understood. We studied the process of assembly of obscurin into nascent sarcomeres during the development of the striated structure of differentiating myofibrils in cell cultures of neonatal and adult rat cardiac myocytes. Confocal microscopy of immunolabeled samples demonstrated intense incorporation of obscurin in the areas of the transition from non-striated to striated regions on the growing tips of myofibrils. Obscurin rapidly and precisely accumulated in the middle of the A-band regions in the open Z-I-band interfaces of half-sarcomeres between newly formed terminal Z-discs and the areas of longitudinally oriented non-striated fibers. This process occurred before the assembly of the second Z-disks on each sarcomere on the tips of growing myofibrils. The presence of obscurin in the areas of the second Z-disks was detected after complete assembly of sarcomeric structure and followed the incorporation of this protein into A-bands. This suggests that obscurin may serve for organization and alignment of myofilaments of the A-bands into the striated pattern. The comparison of obscurin, titin and actin localization in these areas showed that obscurin concentrates into A-bands soon after the incorporation of titin and actin. Electron microscopy of growing myofibrils showed intense formation and assembly of myosin filaments into the Z-I complexes of the terminal nascent sarcomeres. Myosin integrated into the "open" half-assembled sarcomeres in the regions of the terminal Z-I structures, which frequently occurred before the formation of the second Z-bands and the development of mature M-lines of differentiated myofibrils. Abundant non-aligned, thin immature sarcomere bundles near the growing ends were spatially separated and acquired the registered pattern in more distal regions of myofibrils. Thus, obscurin is directly involved in the patterning and alignment of sarcomeres into the register at advanced stages of myofibrillogenesis.

48. The Changing Mechanial Environment Of The Developing Atrioventricular Cushions

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Objectives: Mechanical signaling may play a role in valvular morphogenesis, but quantifying the hemodynamic and tissue strain environment has been limited by the extreme speed, small size, and constantly changing shape of the developing cushions/leaflets. Methods: To overcome these limitations, Micro-CT (VivaCT, Scanco, inc.) was used to quantify the three-dimensional lumen structure of the chick embryonic atrioventricular (AV) canal and left AV (mitral) cushions/leaflets during the post-EMT period (HH17-HH25) at 10 μm voxel resolution. This was combined with in vivo blood flow and cushion motion measurements of ex ovo cultured chick embryos using 55 MHz ultrasound (Vevo660, Visualsonics, inc.) followed by computational fluid dynamics (CFD) simulations using non-Newtonian blood viscosities. Cushion mechanics were measured using a custom-built tissue aspiration system. Results: The CFD models estimate extremely low Reynolds numbers with shear stress regions associated with specific downstream morphogenic changes. AV cushion rigidity increased with stage and was associated with hemodynamic stress. Changes in cushion deformation patterns suggested a transition in AV pumping function. Conclusions: Correlations between mechanical parameters and morphological changes suggest that these forces may play a significant role in shaping embryonic valves, which has important implications for the diagnosis and repair of congenital valve defects and for the mechanical conditioning of living engineered valvular replacements.

49. L-type Calcium Channel (LTCC) Function Contributes to Transcriptional Regulation of the LTCC Revealing a Critical Role of LTCC Function in Heart Development

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Embryonic days 10-12 are a critical time for cardiac morphogenesis. Our working model suggests that the L-type calcium channel (LTCC) is an initiator of signaling that contributes to folding, migration and septation of the developing heart. In contrast to the classical LTCC function as a regulator of contractility, we postulate that LTCC contributes Ca that is utilized for cell signaling. In this study we test the specific hypothesis that LTCC blockade disrupts E10 cardiac morphogenesis independent of the classical role of LTCC regulation of intracellular Ca stores. Whole-heart explants cultured for 48 hours showed gross features consistent with septation, whereas sustained pharmacological LTCC blockade (LTCCB) resulted in the formation of grossly aberrant spheroid-shaped bodies. LTCCB initially stopped spontaneous beating; surprisingly, however, beating resumed within 24 hours despite continual presence of drug block. To probe possible mechanisms we performed calcium imaging and microarray analysis of E10 myocytes in vehicle versus sustained LTCCB. Calcium imaging with fura-2 was used to measure trans-sarcolemmal calcium entry, sarcoplasmic reticulum (SR) calcium, and SR calcium leak in E10 sinus venosus and left ventricular cardiomyocytes. The main finding was that sustained LTCCB increased diastolic trans-sarcolemmal Ca entry. To explore consequences of this increased Ca-entry we performed microarray studies. Microarray analysis showed that LTCCB up-regulated CaV1.2, the main pore-forming subunit of LTCC. Up-regulation of LTCC gene expression is consistent with the paradoxical increase of SL Ca-entry in response to sustained LTCCB. We conclude that sustained LTCCB induces a compensatory up-regulation of LTCC that effects cell signaling crucial for normal heart development.

50. Lmp4 regulates Tbx5 during zebrafish heart development

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The T-box transcription factor Tbx5 has been shown to play crucial roles in vertebrate heart and forelimb development. In humans, mutations in the TBX5 gene lead to Holt-Oram syndrome, a disease characterized by heart and arm defects. In zebrafish, the tbx5 heartstrings (hst) loss-of-function mutation results in embryos that fail to develop pectoral fins (forelimbs) and form a string-like heart. However, there is little understanding of how Tbx5 functions or is regulated. We have identified a novel chicken Tbx5 binding protein, Lmp4, which regulates the nuclear/cytoplasmic localization and transcriptional activity of Tbx5 in cells. Lmp4 dynamically binds to Tbx5, sequestering the transcription factor to actin filaments and thereby modulates its transcriptional activity. However, nothing is known about the function of Lmp4 during development, particularly during cardiogenesis. Here we report the identification of the zebrafish lmp4 homolog, which is expressed along with tbx5, in the developing heart. Inhibition of Lmp4 function by injection of morpholinos (MO) targeting lmp4 protein initiation or splicing resulted in a failure of cardiac looping, reminiscent of tbx5 hst/hst mutants. lmp4 MOs that delete the Tbx5 binding domain also result in cardiac defects, suggesting the in vivo interaction of Tbx5 and Lmp4 is necessary for heart formation. Interestingly hst/+ heterozygous embryos develop normally. However, injection of lmp4 mRNA into hst/+ embryos resulted in cardiac looping defects. Only the the wild-type copy of Tbx5 in hst/+ embryos can interact with Lmp4. Therefore misexpression of lmp4 may increase cytoplasmic wild-type Tbx5 and decrease nuclear Tbx5 causing the heart phenotype. Both lmp4 down-regulation and up-regulation studies support the cellular mechanism of Tbx5 transcriptional regulation and suggest that a balance of nuclear and cytoplasmic Tbx5 is required in the heart. This study shows the first in vivo functional evidence for Tbx5/Lmp4 interactions and nuclear/cytoplasmic shuttling of Tbx5.

51. The role of FKBP12 in endocardial mediated regulation of ventricular trabeculation and compaction

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Ventricular trabeculation and subsequent compaction are important cardiac morphogenetic processes and are critical to the formation and function of ventricular wall. Hypertrabeculation and noncompaction are two main characteristics of a unique type of congenital heart defect-Noncompaction of the Left Ventricular Myocardium (NLVM). Despite its essential role in cardiac formation, the molecular mechanism/s by which regulates these developmental events is largely unknown. One of the best known mouse models for cardiac hypertrabeculation and noncompaction is the mutant mouse deficient in FK506 binding protein 12, which mimics almost every aspect of NLVM. It has been an assumption in the past years that hypertrabeculation and noncompaction are cardiomyocyte-autonomous defect. However, our initial attempt to rescue FKBP12-deficient mice using cardiomyocyte-restricted Fkbp1 transgenic mice was failed, suggesting that cardiomyocyte is not the primary cause of cardiac hypertrabeculation and noncompaction in FKBP12-deficient mice. To further test this idea and determine which cell type in the developing heart is the key to the cardiac trabeculation and compaction, we generated a series of Fkbp1 conditional knockouts by crossing FKBP12flox mice to cell type restricted cre-transgenic mice, including cardiomyocyte specific (FKBP12ckomc), endothelial specific (FKBP12ckoen), and neural crest cell specific (FKBP12ckonc) mutant mice. While FKBP12ckomc and FKBP12ckonc mice have normal ventricular trabeculation and compaction, FKBP12ckoen mice largely recapitulate FKBP12-deficient mice with severe hypertrabeculation and noncompaction and die between E14.5 to birth. This finding strongly suggests that the endocardial endothelium, but not the developing myocardium, is essential in controlling the ventricular trabeculation and compaction. Interestingly, there is no obvious histological defect in endothelial development, which further hints that the intercellular crosstalk between endocardial endothelium and trabecular myocardium is altered in FKBP12-deficient and FKBP12ckoen mice. Our current efforts have dedicated to dissecting molecular pathways in this intercellular crosstalk.

52. CASTOR is required for early cardiogenesis in Xenopus

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The cardiac mesoderm is conceived to be a uniform population of cardiac progenitors. Upon fusion of the cardiac progenitors at the ventral midline, these cells receive molecular cues to undergo differentiation. To date, the molecular pathways that mediate the initiation of cardiac differentiation into different cardiac cell types are poorly understood. We have identified a homologue of Drosophila Castor, a zinc finger transcription factor in Xenopus laevis and Xenopus tropicalis. Drosophila Castor has been shown to be one of the last transcription factors expressed by late-born neuroblasts along the dorsal midline prior to their differentiation, thereby specifying neuronal cell fate. We have cloned and characterized Castor (Cst) from Xenopus and have shown by RT-PCR and in situ hybridization that Cst is expressed in the developing heart and CNS. We depleted Cst from Xenopus embryos and show a fundamental requirement for CST within cardiomyocyte for proper differentiation along the ventral midline. These defects are not due to endodermal defects and results in cardiac morphological abnormalities including cardiac bifida. Collectively, our results suggest that CST mediates regional differentiation processes within the cardiac progenitor population and is essential for cardiogenesis.

53. Atrioventricular Conduction System Development during Late Embryonic and Postnatal Stages
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The atrioventricular (AV) conduction system is required for the propagation of electrical impulse from the atria to the ventricles. The molecular mechanisms underlying the initial formation of the AV conduction system are starting to emerge. Little is known, however, about its subsequent maturation. Here, we did a systematic histological analysis of the AV conduction system in the hearts from embryonic (E15.5, E18.5) and neonatal (N1.5, 1-2 w) CCS-LacZ mice, which express LacZ in the cardiac conduction system. Several components of the extracellular matrix (ECM) were expressed differentially in the AV conduction system compared to the surrounding myocardium throughout development. The AV node was characterized by abundant collagen V expression, and the AV bundle and branches by versican expression. N-cadherin, which promotes cellular adhesion, was present in the chamber myocardium, but barely detectable in the AV node. Interestingly, in embryos and 1-day postnatally, alpha-smooth muscle actin was expressed in the chamber myocardium but not in the AV conduction system. One week after birth, however, this pattern was inverted. Alpha-smooth muscle actin was expressed in the AV bundle and no longer in the remainder of the myocardium. This switch was concomitant with the appearance of collagen I around the AV bundle, and fibronectin at the base of the AV bundle. This postnatal expression of ECM to insulate the AV conduction system from the surrounding myocardium coincides with the postnatal ECM remodeling in AV valves that we reported previously. These data suggest that the development of the AV node and AV valves within the AV canal are spatially and temporally related events and are intriguing given that AV conduction abnormalities can be associated with AV valve defects, such as in patients with Ebstein's anomaly or Holt-Oram syndrome.

54. Functioning Engineered Cardiac Tissue from Skeletal Muscle Derived Stem Cells
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The limited capacity of the injured myocardium to reactivate cardiomyocyte regeneration is a major barrier to the restoration of cardiac function. Numerous types of stem cells are under investigation as alternative cell sources for functioning cardiomyocyte replacement. Here we describe our initial success in differentiating functioning cardiomyocytes from rat skeletal muscle derived stem cells (MDSCs) using MDSC aggregation and three-dimensional engineered cardiac tissue (ECT) culture techniques. MDSCs (pre-plate 6 fraction, PP6) were isolated via the preplate technique from neonate Lewis rat hind-leg muscles. Isolated MDSCs were passaged at least 3 times and underwent rotation culture for 24 hours to form MDSC spheres. MDSC spheres were then mixed with acid soluble rat-tail collagen type I and matrix factors, and cylindrical ECTs were constructed. Spontaneously beating cells were found by culture day 7 and spontaneous tissue contraction by culture day 9. MDSC-ECT expressed Nkx-2.5 and cardiac myosin heavy chain genes. Confocal microscopy identified elongated cells aligned to the ECT longitudinal axis in the region of beating cells. These cells expressed cardiac specific troponin-T and Nkx2.5 proteins. Biomechanical testing revealed that the MDSC-ECT generated contractile force similar to ECT generated from rat embryo cardiac cells. Our preliminary results showed that rat PP6 MDSCs differentiated into a functioning cardiomyocyte phenotype using rotation culture and ECT culture techniques.

55. p38 MAP-kinase regulates engineered early embryonic cardiac tissue proliferation and contractile properties

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The developing fetal myocardium has a high rate of cell proliferation activity which rapidly declines after birth. The loss of cell proliferation activity in the post-natal myocardium is the major barrier to the myocardial regeneration of injured myocardium. Studies have shown that p38 mitogen-activated protein kinase (p38MAPK) regulates cell proliferation, growth, and survival in both developing fetal and post-natal myocardium. We have recently developed engineered early embryonic cardiac tissue (EEECT). EEECT cell proliferation activity and contractile properties mimic native developing myocardium. In the present study, we tested the hypothesis that p38MAPK activity regulates EEECT cell proliferation and contractile properties. Hamburger-Hamilton stage 31 chick embryonic cardiac cells were isolated and mixed with acid soluble collagen type-I and extracellular matrices, and the cylindrical EEECT was constructed. EEECT was cultured until day 10 and 0.5Hz, 5% uniaxial cyclic mechanical stretch was applied for an additional 48 hours. EEECT cell proliferation, contractile force, and p38MAPK and Akt phosphorylation were evaluated. Cyclic mechanical stretch stimulation increased EEECT cell proliferation and contractile force. Inhibition of p38MAPK with a selective inhibitor, SB202190, reduced EEECT cell proliferation and contractile force. The p38MAPK and Akt activities were increased by cyclic mechanical stretch and decreased by p38MAPK inhibition. The positive mechanical stretch effects on EEECT cell proliferation and contractile force were abolished by additive p38MAPK inhibition. Our results suggest that p38MAPK activity plays a key role in the regulation of EEECT cell proliferation and contractile properties.

56. Coronary Vessel Development is Dependent on the Type III Transforming Growth Factor Beta Receptor

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Transforming Growth Factor Beta (TGFβ) Receptor III (TGFβR3) or betaglycan binds all three TGFβ ligands and inhibin with high affinity but lacks the serine/threonine kinase domain found in the Type I and Type II receptors (TGFβR1, TGFβR2). TGFβR3 facilitates signaling via TGFβR1/TGFβR2 but also has been suggested to play a unique and nonredundant role in TGFβ signaling. Targeted deletion of Tgfbr3 revealed a requirement for Tgfbr3 during development of the coronary vessels. Coronary vasculogenesis is significantly impaired in null mice with few vessels evident and numerous, persistent blood islands found throughout the epicardium. Tgfbr3 null mice die at E14.5, the time when functional coronary vasculature is required for embryo viability. However, in null mice nascent coronary vessels attach to the aorta, form two coronary ostia, and initiate smooth muscle recruitment by E14. Analysis of earlier developmental stages revealed defects in the epicardium. At E13.5 these defects include an irregular and hypercellular epicardium with abundant subepicardial mesenchyme and a thin compact zone myocardium. Tgfbr3 null mice also displayed other defects in coronary development including dysmorphic and distended vessels along the AV groove and subepicardial hemorrhage. In null mice, vessels throughout the yolk sac and embryo form and recruit smooth muscle in a pattern indistinguishable from heterozygous or wild type littermates. These data demonstrate a requirement for Tgfbr3 during coronary vessel development that is essential for embryonic viability.

57. MicroRNA Function Is Required for Second Heart Field Development
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The endonuclease, Dicer, processes small RNA precursors into mature ~20-22 nucleotide microRNAs (miRNAs) that control gene expression at the level of mRNA degradation and translational repression. The genome encodes hundreds of evolutionarily conserved miRNAs that can postranscriptionally repress thousands of genes. However, little is known about individual miRNAs that regulate developmental events. To address important miRNAs involved in cardiac morphogenesis, we created a tissue-specific deletion of Dicer, using the Islet1-cre transgenic mouse. The disruption of miRNA biogenesis in the second heart field (SHF) results in early embryonic lethality due to hypoplasia of the right ventricle and abnormal outflow tract formation. We identified a specific miRNA enriched in the SHF and have defined the cis-acting region that directs expression of this miRNA in the SHF. Our data suggest that this miRNA regulates the Wnt/b-catenin pathway during early SHF cardiac progenitor differentiation. Together, these results demonstrate that miRNA processing and function is required for second heart field contribution to the heart.

58. Potassium Channel Subunits Expression Demonstrates a Shift Into a Fetal Gene Expression Programme in a Cardiomyopathic and Arrythmogenic Mouse Model
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Over the last years there are increasing evidence that regulation of the potassium currents is dependent on a wide range of interaction between distinct alpha and beta subunits. IK current seems to be generated by the protein products derived form the Kcnq1 and Kcnh2 genes, in interplay with Kcne1 to Kcne5 beta subunits. In vitro experiments have demonstrated that each beta subunit is able to modulate the current properties generated the by Kcnq1 and Kcnh2 pore-forming subunits in distinct manners. Interestingly, recent data have revealed that all of these beta subunits are transcriptionally expressed in the adult human heart. However, it is unclear how these distinct beta subunits are expressed in the myocytes, and whether there are variations on their expression profiles during distinct developmental and/or physiopathological conditions. We have assessed by quantitative RT-PCR the developmental profiles of the alpha and beta subunits during normal cardiogenesis and in a murine model of dilated cardiomyopathy. We have observed that kcnq1 and kcnh2 display a similar expression profile during atrial and ventricular myocardial development. In contrast, beta subunits display a similar profile during atrial development, being Kcne3 the most represented subunit, whereas Kcne3 is replaced by Kcne2 in the ventricular in adult stages. Dilated cardiomyopathy results in moderate increase of both alpha subunits in the atrial chambers but a significant downregulation in the ventricles. Expression of all beta subunits, except Kcne5, is significantly downregulated in the atrial chambers, whereas Kcne1 and Kcne4 are downregulated and Kcne2, Kcne3 and Kcne5 are upregulated in the ventricular myocardium. Interestingly, the most represent beta subunit is not altered in the atrial myocardium whereas there is a shift in the ventricular myocardium in the dilated cardiomyopathic hearts. These data demonstrate for the first time that dilated cardiomyopathy reprogrammes electrical circuitry towards the embryonic gene expression programme.

59. The Genetic Architecture of Congenital Heart Disease
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Several dozen genes have known roles in cardiac morphogenesis, but how they and unknown genes fit into networks that lead to naturally occurring heart defects is unclear. We thus have taken an unbiased genetic approach to show how modifier genes and their interactions affect the development of cardiac malformations in the presence of Nkx2-5 mutation. Human mutations of the cardiac transcription factor Nkx2-5 cause a broad spectrum of heart defects with variable penetrance. Some heterozygous Nkx2-5 knockout (Nkx2-5+/-) mice on a C57Bl/6 background also develop serious defects like aortic coarctation

and VSD, causing neonatal death. Inbred strain crosses reveal that genetic modifiers influence the Nkx2-5 cardiac phenotype and penetrance. F1 progeny of Nkx2-5+/- C57Bl/6 mice crossed to either of two inbred strains have a nil or rare incidence of defects, but the F2 progeny of the intercross and parental backcrosses again have defects. VSD and ASD are the first and second most common defects in the Nkx2-5+/- F2 animals, and common atrioventricular canal defects are uniquely found in one of the crosses. Linkage analyses for VSD susceptibility loci have identified a potential main effect locus and 11 significant epistatic interactions involving 16 loci, most of which are not near known cardiac developmental genes. The discovery of loci and interactions that influence the development of VSD in the presence of a major gene mutation demonstrates the critical roles of genetic heterogeneity and epistasis in the pathogenesis of congenital heart disease. The results in addition offer mechanistic insights into the commonly observed but puzzling observations of incomplete penetrance and pleiotropy.

60. Identifying Post-Translational Modifications of the Transcription Factor TBX5 that Function During Heart Development

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TBX5, a T-box transcription factor, plays critical roles during vertebrate heart development. Mutations in TBX5 result in Holt-Oram syndrome, a dominant autosomal human disorder characterized by congenital heart and upper limb defects. To determine the function of Tbx5 during cardiac development we depleted TBX5 in Xenopus laevis. Our studies show that Tbx5 depletion results in cell cycle arrest at the G1/S transition, fewer cardiac cells, alterations in cardiac differentiation, and ultimately malformation of the heart. Despite this critical role in heart development little is understood about how TBX5 exerts its control over this process. Tissue culture studies suggest that TBX5 has interactions with multiple protein partners. However, the mechanisms of specificity and temporal control of these interactions in vivo is unknown. Preliminary studies in our lab suggest that post-translational modifications may provide a method for regulating TBX5-protein interactions during cardiac development. Using Xenopus, we are isolating TBX5 from embryos at critical developmental time points during early heart formation. TBX5 will then be subjected to 2-D gels, western blot, and mass spectrometry for determination of post-translational modifications based on known changes in mass for modifications such as phosphorylation and methylation.

61. Defects in ciliary structure and function is associated with laterality defects in a novel Mdnah5 dynein mutation

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The specification of left-right asymmetry is essential for the formation of the four chamber heart and elaboration of separate systemic and pulmonary circulation critical to survival of air breathing mammals. Previous studies suggest motile function associated with the monocilia at the embryonic node plays an essential role in left-right patterning. We recovered a mouse mutation consisting of a deletion of sequences from the N-terminus of mouse heavy chain dynein Mdnah5, spanning a region containing a putative dynein interacting domain likely critical for ciliary function. Homozygote mutants exhibit situs solitus, situs inversus totalis or heterotaxy syndrome with complex structural heart defects, phenotypes consistent with primary ciliary dyskinesia. MicroCT scanning showed homozygote animals surviving up to five weeks with situs solitus or situs inversus eventually died from severe hydrocephaly, while those dying much earlier showed complete blockage of the airway passages. These phenotypes likely reflect ciliary dysfunction. Videomicroscopy of the ciliated airway epithelia showed homozygous mutants have immotile or slow dyskinetic ciliary motion, while in heterozygote animals, we observed rapid synchronous beating to dyskinetic or absent ciliary motion. Quantitation of ciliary function using fluorescent beads show reduced net flow in homozygote animals, but surprisingly, no difference in net flow was observed in heterozygote vs. wildtype animals. Ultrastructural analysis of the airway epithelia by electron microscopy revealed largely absent outer dynein arms (ODAs) in the cilia of homozygote

mutants, while heterozygous animals show variable phenotypes that range from normal ODA distribution to absent ODA. Using observations from the airway epithelial as a proxy for nodal ciliary function, we propose left-right specification may be critically dependent on net flow achieved at the embryonic node. Further studies of ciliary motion in the embryonic node using high resolution digital videomicroscopy are being pursued to elucidate the precise role of the nodal cilia in left-right specification.

62. Bmp2 Gene Regulation in the Heart & Vasculature

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Bone Morphogenetic Protein (BMP)2 is an essential cardiac morphogen whose levels must be precisely controlled in the developing heart and vasculature. Later in adults, BMP2 up-regulation initiates pathological calcification of blood vessels and heart valves. Therefore, identifying the mechanisms that regulate BMP2 synthesis is relevant to both normal heart and valve development and to how vascular and valve cells are diverted to osteogenic fates. Several hundred nucleotides within the 3' untranslated regions (UTRs) of BMP2 genes from mammals to fishes are extraordinarily conserved over 450 million years of separation indicating that the region is under stringent selective pressure. Ubiquitously expressed, CMV-driven Cre-recombinase caused excision of this ultra-conserved sequence from our novel BMP2-controlled reporter transgene model. This deletion dramatically induced reporter gene expression in many cell types including the aorta, pulmonary trunk, coronary arteries, heart valves and lungs where BMP2 is not normally expressed in the adult, except in pathological situations. These in vivo results together with tissue culture results suggest that the ultra-conserved sequence represses BMP2 expression in some cell types. Our in vitro and tissue culture analyses indicate that this region controls BMP2 expression by post-transcriptional mechanisms. We will present our data indicating that our reporter gene mimics many aspects of normal BMP2 regulation, possibly including BMP2 induction in response to high fat diet, and that the ultra-conserved region represses BMP2 expression in specific tissues in embryos and adults.

63. Ets-1 Deficient Mice Die Perinatally due to a Membranous Ventricular Septal Defect

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Heart formation is a finely regulated process with mutations of key cardiac transcriptional factors resulting in congenital heart disease. The Ets transcription factor family, characterized by a highly conserved DNA binding domain, consists of more than 30 members. Ets family members have been shown to be involved in broad spectra of biological processes; however, little is known about Ets function in heart development. Here we investigated the role of Ets-1, the founding member of Ets family, in cardiac development. In situ hybridization of mouse embryos demonstrates that at embryonic day 12.5, Ets-1 is highly expressed in the epicardium and endocardium, but not expressed in the myocardium. Previously, Ets-1 deficient mice were reported to have an incompletely penetrant perinatal lethal phenotype on a mixed genetic background, although the cause of this lethality was never investigated. When crossed to C57BL/6 background, this phenotype became nearly completely penetrant, with Ets-1 deficient mice dying shortly after birth. Histological examination of Ets-1 deficient embryos revealed a membranous ventricular septal defect, the likely cause of perinatal lethality. These mice were also found to have an abnormal focus of cartilage in the heart. This cartilage was first detected between embryonic day 14.5 and 16.5 in the peri-aortic area. Consistent with this observation, the cartilage lineage marker Sox9 was up-regulated in Ets-1 deficient mice in this region. Previous studies have demonstrated that MEK-ERK pathway directly regulates the activity of Ets-1. To determine if this pathway is involved in the regulation of Ets-1 and cartilage formation in the heart, we applied MEK inhibitor U0126 to a whole heart organ culture system. We found that U0126 not only up-regulated Sox9 mRNA level, but also caused aberrant cartilage formation, suggesting the involvement of MEK-ERK-Ets-1 pathway in repressing abnormal cartilage formation. Taken together, these results suggest the importance of Ets-1 for normal heart development as a negative regulator of cartilage formation.

64. Potential role of obscurin-like 1, OBSL1, in the assembly of the cardiac intercalated disk

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Obscurin-Like 1, OBSL1, is a novel protein that localizes to the intercalated disks (IDs), the Z bands and the perinuclear region of mature cardiac myocytes. To determine OBSL1's potential functions, its cellular localization pattern was examined in primary cultures of adult rat cardiac myocytes. Under appropriate culture conditions, these myocytes dismantle their contractile elements, spread, and re-establish cell-cell and cell-matrix contacts, subsequently assembling new myofibrils and reorganizing their intracellular cytoskeleton in a manner that closely recapitulates the in vivo processes that occur during development. As myocytes began to remodel, OBSL1 rapidly dissociated from the Z band, well before the disassembly of the contractile apparatus. It was more gradually removed from the IDs, eventually redistributing diffusely along the cell periphery. There it co-localized with β1-integrin as the cells spread and made new contacts with neighboring cells. As these contacts matured, β-catenin and connexin43, critical adherens junction and gap junction components respectively, were recruited to specific cell contact sites which became organized into new ID-like structures. In contrast to its very early localization to organizing cell-cell contacts, OBSL1 did not distribute to the area overlying the Z bands until well after the assembly of new myofibrils and the re-organization of the intermediate filament system, suggesting that OBSL1 may instead be associated with the overlying membranes of the sarcoplasmic reticulum (SR), the last compartment to reestablish its mature organization in this model. Based on these studies, OBSL1 appears to be a novel cytoskeletal linker that is closely associated with the sub-membrane cytoskeleton and may participate in the organization and stabilization of the cardiac ID and other cell-cell and membrane-cytoskeleton linkage complexes. Further characterization of OBSL1 may contribute to our understanding of the assembly of these structures during cardiac development and allow the design of strategies to preserve their integrity during pathophysiologic stress

65. Retinoid-BMP-GATA signaling network during avian cardiogenesis

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Vertebrate heart formation is a complex process and is a result of many intracellular signaling pathways as well as tissue interactions. Events that disturb this process may result in congenital heart defects. Signaling factors that play a role in cardiac development include retinoic acid (RA; vitamin A), bone morphogenetic proteins (BMPs), and GATA family transcription factors. RA regulates the development of the heart and foregut. Excess RA and vitamin A deficiency (VAD) during embryogenesis produces cardiac and gut defects. We found that decreased levels of GATA4 in cardiogenic mesoderm and foregut endoderm correlate with the VAD associated heart and foregut abnormalities, but that this could be rescued by transplanting normal anterior endoderm. We also found that (like GATA4) transcripts encoding BMP2 and BMP-dependent signaling activity are decreased throughout the heart-forming region of the VAD embryo. Addition of BMP2 protein or forced expression of GATA4 in VAD embryos leads to a partial rescue of the VAD phenotype, and addition of both BMP2 and GATA4 has an additive positive effect. Our data are consistent with a requirement for retinoid signaling to maintain expression of BMP2, which regulates GATA4 expression, and in addition acts, with GATA4 to regulate genes important for normal morphogenesis of the primitive heart tube and foregut.

66. Zebrafish as a Model for Environmental Contaminant-Induced Hypoplastic Left Heart Syndrome

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Hypoplastic left heart syndrome (HLHS) is a devastating congenital cardiovascular malformation of unknown etiology. In a recent epidemiological study, a cluster of HLHS occurring in the Baltimore-Washington area has been linked to the environmental release of dioxins and polychlorinated biphenyls (PCBs). These compounds are known to act through the aryl hydrocarbon receptor (AhR), but the mechanisms by which the AhR pathway elicits its toxic effects are poorly understood. Having previously shown evolutionary conservation in the cardiac outflow tract of the zebrafish, we are utilizing this species and a model PCB (PCB126), to investigate mechanisms of toxicity of such compounds. We found that cardiac tissues are specifically targeted in early development, leading to a phenotype we describe as 'stringy heart'. Prior to 24 hours post-fertilization (hpf), cardiac markers appear unaffected by PCB126 exposure, but by 72hpf, exposed fish exhibit extreme pericardial and yolk-sac edema, jaw malformations, a stretched, 'stringy' heart, a severely reduced bulbus arteriosus and diminished or absent blood circulation. By performing cell counts, we have found that, similar to cases of HLHS, PCB126 specifically affects ventricular, but not atrial, myocardium. Between 36 and 72hpf, there is a marked reduction in proliferation in affected tissues, as shown by BrdU incorporation. The lack of a corresponding increase in apoptosis suggests cell-cycle arrest. This led us to hypothesize that the tumor suppressor protein, p53, acts downstream of the AhR to induce cell-cycle arrest and senescence. We have subsequently shown that inhibition of p53 activity, using a chemical suppressor, pifithrin-_, rescues the 'stringy heart' phenotype. Furthermore, we have found that levels of p53 protein are elevated following PCB126 exposure. This work validates the use of zebrafish for the study of environmental contaminant-induced HLHS and is the first report of a potential mechanism by which activation of the AhR may lead to cardiac-specific toxicity.

67. Expression profiling in the hearts from wild-type, heterozygous, and mXinalpha-deficient mice

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Targeted deletion of mouse mXinalpha gene results in abnormal intercalated disc ultrastructure, cardiac hypertrophy, cardiomyopathy and conduction defects. To understand the molecular mechanism leading to these defects, we have examined expression changes for most intercalated disc proteins by Western blot analysis, shown changes in the message levels for some known channel components by qRT-PCR and shown changes in channel activities by patch-clamp electrophysiological studies. However, these results only indicate changes in the intercalated disc structural integrity and in channel activity which does not provide information about signaling and/or pathways leading to these cardiac defects. Genome-wide expression profiling with microarray hybridizations was used to identify gene expression changes in hearts lacking mXinalpha. For each genotype (wild-type, heterozygote, knockout), total RNA was isolated from 4 hearts of 9-month old mice and used to generate cDNA labeled with the 3DNA Dendrimer labeling technology. This cDNA was hybridized to a mouse microarray slide featuring 15,436 nonredundant genes printed in duplicate. Each scanned slide image was normalized, had features flagged for quality, and then had a GPR file generated with GenePix Pro6.0. Statistical significance for the fold change of each transcript between genotypes was determined in Genespring 7.3.1GX using a standard one-way ANOVA. Then a pair-wise comparison of each genotype was performed for those transcripts with a $P \leq 0.05$ that generated a list of 496 genes with a significant fold change in expression. Among these genes, 367 encode known proteins with some characterized function, 53 encode known proteins

without a characterized function, and 76 genes are unknown. When the genes are organized by gene ontology, several interesting clusters are apparent for Wnt/cadherin, JNK, G-protein, and calcineurin signaling as well as cell cytoskeleton and adhesion. Some genes identified in these cluster have been selected for further validation by RT-PCR, in situ hybridization and/or immunofluorescence microscopy.

68. Role of Cited2 in AV endocardium EMT during heart development

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Cited2 (CBP/p300-interacting transactivator, with Glutamic acid (E)/Aspartic acid (D)-rich carboxy-terminal domain, 2) is a transcription co-activator, which strongly interacts with CBP/p300 but has no identifiable DNA binding domain. Cited2 protein expression can be activated by cytokine treatment, low oxygen tension, lipopolysaccharide and shear stress. Targeted deletion of the Cited2 gene in mice leads to defects in multiple organs, including heart, brain, lung, liver, eye, placenta, and spleen. The heart defects observed in Cited2-deficent mice include ventricular septal defects (VSDs), atrial septal defects (ASDs), AV junction defects, outflow tract defects, aortic arch defects, and coronary vessel abnormalities. It was reported that cell density in the AV cushion at E10.5 stage is lower in Cited2 knockout embryos. Since we showed that Cited2 enhances TGF-β mediated EMT in both human breast cancer cells (MDA-MB-231) and mouse mammary epithelial cells (NMuMG), we hypothesized that Cited2 is important for AV endocardium EMT. First, we confirmed that the density of mesenchymal cells in the AV cushions is decreased in the Cited2 knockout mice, at E10.5 stage. We also found that this decrease persisted through E11.5 making it unlikely that Cited2-deficient embryos are merely developmentally retarded because ventricular trabeculae were correctly developed by E11.5 stage. Neither proliferation, as detected by phosphorylated-histone H3 immunostaining, nor cell death, detected by the TUNEL assay, were significantly different in the E10.5 stage Cited2 knockout embryos compared to wild-type littermates. Our results indicate that Cited2 deficient mice may have a reduced number of mesenchymal cells in the AVC due to a defect in the EMT process. Immunostaining and real time RT-PCR will be performed to evaluate the expression of EMT markers and to explore potential pathways that might contribute to the defective EMT process in Cited2 deficient AV cushions. (This work was supported by NIH RO1HL75436).

69. Loss of mXinalpha, a beta-catenin-interacting and actin-bundling protein, in mice results in cardiomyopathy with conduction defects

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The intercalated disc protein, Xin, was originally discovered in chicken heart and implicated in cardiac morphogenesis. In the mouse, there are two homologous genes, mXinalpha and mXinbeta. mXinalpha-null mouse hearts are hypertrophied and exhibit fibrosis. The amount of fibrosis detected in the mXinalpha-deficient mice increases with age from 3 to 14 months. Ultrastructural studies of mXinalpha-null hearts from 3 month-old mice reveal intercalated disc disruption and myofilament disarray. In mXinalpha-null mice, there is a significant decrease in the expression level of p120-catenin, beta-catenin, and N-cadherin, which could compromise the integrity of the intercalated discs and functionally weaken adhesion, leading to cardiac defects. Additionally, altered localization and decreased expression of connexin 43 is observed in the mXinalpha-null mouse heart, which could lead to conduction defects. Indeed, ECG recordings on the isolated perfused hearts (Langendorff's preparations) show a significantly prolonged QT interval in mXinalpha-deficient hearts. To understand the underlying mechanisms leading to such cardiac defects, we have demonstrated mXinalpha directly interacting with beta-catenin and

mapped the beta-catenin binding site to amino acid #535-636, which overlaps with the actin-binding domains composed of the Xin repeats. Purified recombinant mXinalpha are capable of bundling actin filaments. Full-length mXinalpha binds actin weakly than an mXinalpha C-terminal deletion mutant, suggesting a model whereby the C-terminus of mXinalpha may prevent the full-length molecule from binding to actin, until the beta-catenin binding domain is occupied by beta-catenin. The binding of mXinalpha to beta-catenin at the adherens junction would then open the stronger binding sites for actin. Supporting this model, we found that the binding of mXinalpha to actin was enhanced two folds in the presence of beta-catenin. Thus, mXinalpha functions in regulating the hypertrophic response and maintaining the structural integrity of the intercalated disc in normal mice, through its interaction with beta-catenin and actin.

70. Embryonic Origins of Cardiac Malformations in the Ts65Dn Down Syndrome Model

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The Ts65Dn mouse, with triplication of fifty percent of the genes found on human chromosome 21 (Hsa21), models the genotype and phenotypes of Down syndrome. We have identified and characterized the cardiac malformations present in these trisomic mice, which are prone to neonatal lethality. Vascular abnormalities, including right-sided aortic arch, were identified in 17 percent of trisomic neonates by examination of gross anatomy. Ventricular septal defect and broad foramen ovale are present in some Ts65Dn pups. Histochemical analyses demonstrate aberrant valvular morphology. Our embryonic studies indicate that the malformations originate during the early stages of cardiac septation. Comparison of the branchial arch arteries of trisomic Ts65Dn and euploid siblings at embryonic day 10.5 (E10.5) demonstrates incomplete or delayed development of the arch arteries. Later stages of septation are also disrupted by the dosage imbalance of the HSA21 orthologs, as apoptosis in the endocardial cushions of trisomic E13.5 embryos is reduced compared to euploid levels. Our results indicate that triplication of key genes, such as DSCR1, DYRK1A, and ADAMTS1, disturbs several different processes that may lead to the observed cardiovascular and intracardiac anomalies in Ts65Dn mice.

71. Echocardiograhic Indices for Murine Heart Valve Structure and Function with Morphometric Histologic Correlation from the Fetus through the Aged Adult

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Ultrasound techniques are ideally suited for in vivo evaluation of the cardiovascular system; and noninvasive echocardiographic assessment of ventricular function in transgenic mice has been established. To study perinatal and postnatal changes in normal and diseased valves, we established normal indices of mouse valve structure and function and correlated these findings with morphometric histologic analyses of the aortic and mitral valves. Echocardiographic studies were performed with a Visual Sonics Vevo 770 imaging system and a 30mHz transducer. C57Bl6 mice of mixed gender were studied at serial time points: embryonic day 18.5 (late fetal), 10 days (newborn), 1 month (juvenile), 2 months (young adult), 9 months (old adult) and 16 months (aged adult). Direct and indirect indices of valve structure and function were measured, including annulus dimensions, flow velocities, chamber sizes and ventricular function. Measurements were made in triplicate from 10-14 mice at each time point. Histochemical analysis using pentachrome stain was then performed to assess ECM organization, and morphometric analysis using ImageJ was used to measure valve thickness, length and area. Measurements were made in three hearts per time point from three sections per heart in triplicate, and these parameters were directly compared with echocardiographic data for the same hearts. Valve function remained normal despite progressive elongation and thinning of valve cusps and leaflets. Histochemical analysis demonstrated measurements ~40% smaller than echocardiographic measurements, consistent with previous findings showing tissue shrinkage due to processing methodology. These findings define normal ranges for echocardiographic indices of murine valve structure and function during normal growth,

development and aging, and provide a reproducible systematic approach for the evaluation of transgenic mouse models of valve malformation and disease.

72. Elastin Haploinsufficiency is Associated with Aortic Valve Malformation and Latent Valve Disease in a Mouse Model

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Elastin is an extracellular matrix (ECM) protein that is a primary component of semilunar and atrioventricular valves and great arteries. Elastin haploinsufficiency is an important clinical problem characterized by supravalvar aortic stenosis, and valve disease is present in 10-20% of cases. Based on findings in a patient with elastin haploinsufficiency and valve disease, we hypothesized that elastin insufficient mice would manifest viable heart valve disease. Mice with a homozygous deletion of elastin (ELN -/-) have severe arteriopathy and perinatal death; the arteriopathy of ELN +/- mice results in systemic hypertension. We used echocardiography to evaluate valve function in vivo in 1, 2 and 18 month-old (mo) heterozygous ELN +/- mice and age-matched wild type controls. Hearts were then harvested and histochemical analysis was performed to evaluate aortic and mitral valve morphology and ECM organization using pentachrome stain. The 1 mo ELN +/- mice demonstrated normal valve function, but valve disease was identified in 1/6 (17%) 2 mo ELN +/- mice (mild aortic regurgitation), and 10/18 (56%) 18 mo ELN +/- mice, including moderate aortic regurgitation (4, regurgitant velocity greater than 2m/s and holodiastolic), mild aortic regurgitation (6), mild aortic stenosis (2, peak gradient greater than 20mmHg) and moderate mitral regurgitation (1). Those with moderate aortic regurgitation showed aortic valve redundancy. There was no right-sided valve disease, but some mice had aortic and mitral valve abnormalities. There was no valve disease in age-matched controls. Histochemical analysis demonstrated thin elongated valves with increased cell density and ECM disorganization as evidenced in part by decreased and fragmented elastic fibers. These findings establish a role for elastin in the pathogenesis of latent valve disease, and provide a mouse model, the heterozygous ELN +/- haploinsufficient mouse, to study underlying mechanisms of viable valve disease.

73. Regulation of TGFβ2 signaling during midgestation heart development by retinoid receptor ligands occurs in a time-frame that is independent of transcriptional events

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A considerable proportion of congenital heart diseases are the result of defects in outflow tract development. While it is known that significant outflow tract remodeling occurs during midgestation and involves cellular proliferation, differentiation and apoptosis, the exact signaling cascades controlling these events are poorly understood. We have previously reported increased TGFβ2 signaling as well as increased apoptosis in the developing outflow tract of the midgestational (E11.5-13.5) retinoid x receptor alpha knockout (RXRα−/−) mouse model of congenital heart disease. Interestingly, Smad2, a signaling cofactor downstream of TGFβ2, is perturbed in the RXRα−/− such that cells within outflow tract mesenchyme of the null show a lack of nuclear-localized, activated Smad2 (pSmad2) when compared to similar cells in the wild type. This apparent paradox led us to investigate whether activation of retinoic acid signaling may affect pSmad2 accumulation. Therefore, we treated wild type E12.5 dispersed whole heart cells with combinations of TGFβ2 and the RXRα ligands, 9-cis-retinoic acid (9-cis-RA) and bexarotene (Targretin, LGD1069), and evaluated activation of Smad2, which occurs within 30-60 minutes of treatment. As expected, treating heart cells with TGFβ2 for 1 hour resulted in an increase in pSmad2 and a decrease in total Smad2 as detected by Western blot when compared to treating with 9-cis-RA, bexarotene or diluent alone. Interestingly, when cells were treated with the combination of TGFβ2 and RA ligands, we detected a statistically significant, synergistic increase in the amount of pSmad2 when compared to treating with TGFβ2 alone. Additionally, co-treatment of TGFβ2 and 9-cis-RA led to a

sustained level of pSmad2 for several hours compared to TGFβ2. Our results suggest that an important and integral component of TGFβ/Smad-mediated events during cardiogenesis is the direct interaction between RXRα and signal molecules in the TGFβ-signal cascade, specifically Smad2. We pose a molecular mechanism to explain these observations.

74. Identification of distal regulatory sequences of the ANF gene that control its developmental activity and re-activation during cardiac disease

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Nppa, the gene encoding Arial Natriuretic Factor (ANF), is specifically expressed in the atrial and ventricular chamber myocardium of the developing heart, and down-regulated in the ventricles around birth. In conditions of cardiac stress Nppa is re-activated in the ventricles as part of a conserved 'fetal' gene program, making it an excellent marker to study gene regulation in cardiac disease. Small promoter fragments of Nppa were reported to drive correct gene expression in transgenic mice and cultured cells. They have become the most frequently used promoters to study cardiac gene regulation in development and during stress. Here we show that 0.7 to 7 kbp Nppa promoter fragments provide correct activity in the atria and nodes, but, unexpectedly, lack ventricular enhancer activity for correct pre-natal expression, and are not induced in response to cardiac disease. Using bacterial artificial chromosomes (BACs) we found that for correct pre-natal ventricular expression two distal sequences are required. One provides the correct pattern (spatial enhancer), while distinct sequences control the level of pre-natal ventricular expression (embryonic ventricular enhancer). Finally, we show that for the induction of Nppa during disease distal regulatory sequences are required that work independently of the embryonic ventricular enhancer. In conclusion, the regulation of Nppa is much more complicated than previously appreciated, with at least two additional distal enhancers providing different parts of the regulation of Nppa during development and disease. The fetal ventricular activity and the re-activation during disease appear to be uncoupled, requiring distinct regulatory sequences. Furthermore, proximal Nppa promoter activity does not reflect ventricular gene regulation and as such cannot be used as a paradigm for cardiac gene regulation. The Nppa-BAC transgenic lines that we generated may provide useful tools in studies of ventricular gene regulation in development and disease.

75. BMP signaling induces mesenchymal cell migration and periostin expression in post-EMT AV valvulogenesis

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Atrioventricular (AV) endocardium transforms into the cushion mesenchyme, the primordia of the valves and membranous septa, through epithelial-mesenchymal-transformation (EMT). Although bone morphogenetic protein (BMP)-2 is known to be critical for AV EMT, the role of BMP-2 in post-EMT AV valvulogenesis remains to be elucidated. To find possible BMP signaling loops, we first localized Type I BMP receptors (BMPRs), BMPR-1A, -R-1B, and ALK2 in stage-24 chick AV cushion mesenchyme. Based on the BMP receptor expression pattern, we examined the functional roles of BMP-2 and BMP signaling in post-EMT AV valvulogenesis by using stage-24 AV cushion mesenchymal cell aggregates cultured on three dimensional collagen gels. Exogenous BMP-2 or constitutively active (ca) BMPR-1B virus treatments enhanced phospho-Smad1/5/8 expression while noggin, an antagonist of BMPs, or dominant-negative (dn) BMPR-1B treatments reduced phospho-Smad1/5/8 expression in cushion mesenchymal cells. BMP-2 or caBMPR-1B induced migration of the mesenchymal cells into collagen gels, whereas noggin or dnBMPR-1B virus treatments reduced cell migration. BMP-2 or caBMPR-1B treatments significantly promoted expression of an extracellular matrix protein, periostin, a known valvulogenic matrix maturation mediator in AV valvulogenesis at both mRNA and protein levels. Periostin expression was repressed by adding noggin or dnBMPR-1B virus to the culture. Transcripts of Twist, and Id1, which have been implicated in activation of the periostin promoter in osteogenesis, were

also induced by BMP-2 but repressed by noggin treatments in cushion mesenchymal cell cultures. Furthermore, microinjection of caBMPR-1B virus induced periostin expression in cardiac cushions while dnBMPR-1B virus repressed periostin expression in vivo (ovo). These data provide evidence that BMP-2 and BMP signaling induce biological processes involved in early AV valvulogenesis, i.e. mesenchymal cell migration and expression of periostin, which indicates critical roles for BMP signaling in post-EMT AV cushion tissue maturation and differentiation. Supported by NHLBI grant HL33756.

76. The neurofibromin GAP-related domain rescues endothelial but not neural crest development in Nf1–/– mice

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Neurofibromatosis type I (von Recklinghausen's disease) is a common autosomal-dominant condition primarily affecting neural crest–derived tissues. The disease gene, NF1, encodes neurofibromin, a protein of over 2,800 amino acids that contains a 216–amino acid domain with Ras–GTPase-activating protein (Ras-GAP) activity. Potential therapies for neurofibromatosis currently in development and being tested in clinical trials are designed to modify NF1 Ras-GAP activity or target downstream effectors of Ras signaling. Mice lacking the murine homolog (Nf1) have mid-gestation lethal cardiovascular defects due to a requirement for neurofibromin in embryonic endothelium. We sought to determine whether the Ras-GAP activity of neurofibromin is sufficient to rescue complete loss of function or whether other as yet unidentified functions of neurofibromin might also exist. Using cre-inducible ubiquitous and tissue-specific expression, we demonstrate that the isolated GAP-related domain (GRD) rescued cardiovascular development in Nf1–/– embryos, but overgrowth of neural crest–derived tissues persisted, leading to perinatal lethality. These results suggest that neurofibromin may possess activities outside of the GRD that modulate neural crest homeostasis and that therapeutic approaches solely aimed at targeting Ras activity may not be sufficient to treat tumors of neural crest origin in NF1.

77. Enhancer of Polycomb1, a new modulator for sarcomeric muscle differentiation

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Enhancer of Polycomb1 (Epc1) is an unusual member of the polycomb group gene family. Epc1 itself does not have enzymatic activity, the complex including Epc1, however, is reported to possess both activating and repressive activities as a transcription regulator. Although homozygotic mutations of Epc1-homolog in Drosophila are lethal in embryo, the biological significance in the mammalian cells has to be identified. Here we report that Epc1 is a novel binding candidate of homeodomain only protein, Hop, a transcriptional modulator that is essential for the normal development of the mammalian heart and that it regulates the skeletal muscle differentiation. Yeast-two hybrid assay with human adult heart cDNA library revealed that the Hop can associate with Epc1. Amino terminal domain of Epc1 as well as full Epc1 physically interacted with Hop in mammalian cells and in yeast. Epc1 was highly expressed in the embryonic heart and adult skeletal muscles. Serum deprivation induced differentiation of H9c2, a myoblast cell line, into skeletal myocytes and Epc1 was transiently upregulated. Differentiation of H9c2 was induced by Epc1-overexpression, while it was severely impaired in Epc1-knockdown cells. Co-transfection of Hop potentiated Epc1-induced transactivation of myogenin and atrial natriuretic factor. Epc1-induced myotube formation as well as decrease in cell survival was further potentiated by co-transfection of Hop. Hop knockout mice elicited decrease in myosin heavy chain and myogenin expressions in skeletal muscle and showed delay in hamstring muscle healing after injury. Differentiation was impaired in skeletal myoblasts from Hop knockout mice. Epc1 physically interacted with serum response factor (SRF) as well as its coactivator, myocardin. Epc1 potentiated SRF-mediated promoter

activations of smooth muscle 22αor atrial natriuretic factor. These results suggest that Epc1 plays a role in the initiation of sarcomeric muscle differentiation.

78. Development of novel cellular and molecular tools for in vivo bioluminescent imaging in the heart

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Bioluminescence holds great promise for in vivo cellular and molecular imaging, especially in small animal models. To explore the potential of bioluminescent imaging for evaluating cell and gene therapies in the mouse heart, we have recently developed several novel tools and techniques. Specifically, we have created new recombinant mouse embryonic stem (mES) cell lines that stably express the firefly luciferase (LUC) reporter gene under the control of different cellular promoters that enable determination of the health and differentiation status of these cells following transplantation into an ischemic mouse heart model. To assess whether mES cells differentiate and/or maintain cardiomyocyte characteristics in vivo, we created recombinant mES cells that stably express LUC driven from the cardiomyocyte-specific alpha-myosin heavy chain (αMHC) promoter. These αMHC-LUC cells preferentially express LUC reporter gene activity following differentiation into beating cardiomyocytes. As a control, we have also developed a constitutive LUC cell line driven by the "housekeeping" glyceraldehyde phosphate dehydrogenase (GAPDH) promoter. Stable recombinant GAPDH-LUC cells express LUC activity in both the undifferentiated (pluripotent) and cardiomyocyte-differentiated states. Transplantion of these promoter-specific recombinant LUC mES cell lines into mouse hearts will enable us to determine both the viability and differentiation status of these cells and allow us to assess their potential for cardiac regeneration/repair therapies. In parallel, we have also used biolistic gene transfer to demonstrate the efficacy of molecular in vivo bioluminescent imaging in the mouse for potential gene therapy applications. Our results show that LUC activity peaked within 2-3 days following transfer of LUC reporter genes using high velocity DNA-gold microparticle bombardment of targeted tissues with a Helios™ "Gene Gun". Thus, the novel tools that we are developing for bioluminescent imaging can be informative for both gene and cell therapy applications.

79. Folbp-1 Murine Model to Study Folic Acid Involvement in Anterior Heart Field Development

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Periconceptional folic acid supplementation can reduce the occurrence of complex congenital defects in humans. In an effort to understand the mechanisms underlying the beneficial effects of folic acid during development, we have developed genetically modified mice whose folate transport systems have been ablated. Inactivation of the folate binding protein-1 (Folbp1) gene results in embryonic lethality by E10. Administration of folinic acid to Folbp1 deficient dams rescues the nullizygous embryos; however, surviving embryos present with malformations involving the neural tube, craniofacies, heart, eyes and abdominal wall. In the absence of adequate maternal folate, there are notable changes in the Folbp1 mutant embryos at both the molecular and cellular levels. Folate binding protein 1 is a GPI-anchored folate transport protein highly expressed in the yolk sac, the maternal placenta as well as the syncytiotrophoblast and fetal neuroepithelium of both the neural plate and contiguous areas that would include presumptive cranial neural crest cells. Examination of the cross sections of the neural fold/tube revealed Folbp1 expression in the roof plate and possibly premigratory cranial neural crest cells during early development. Outflow tract defects are commonly detected in Folbp1 nullizygotes whom received only modest folate supplementation in utero. In an attempt to understand the developmental consequences of abnormal folate transport on the molecular level, we initiated a series of whole mount in situ hybridization studies. Preliminary results show a decreased expression of selected Wnt genes, Isl1, the neural crest marker PlxnA2 expression in the pharyngeal arches and anterior heart field areas of nullizygous treated with 6.25 mg/kg of SFA. Given the involvement of Wnt signaling in the Folbp1 knockout embryos, subsequent studies will be conducted where embryonic cell will be harvested and treated with folic acid to better understand the impact of the Wnt signaling pathway and folic acid to the

expression of anterior heart field genes in this mouse model. Supported in part by grants HL66398 and HL085859

80. Prox-1 in the Avian Embryonic Heart

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A critical function of the lymphatics is to regulate the fluid balance of the internal environment. The lymphatics of several adult organ systems including the heart have been described in detail, but this is not the case for the embryonic heart. We investigated the lymphatics of the avian embryonic heart by immunostaining whole quail and chicken hearts for Prox-1, which has been linked to lymphatic development and cellular differentiation. Anti-human Prox-1 has been reported to identify quail lymphatic endothelial cells during lymphangiogenesis in chorioallantoic membranes. Consistent with this finding, cells with nuclear-localized Prox-1 were found organized into vessels distinct from blood vessels at the end of ventricular septation (H&H stage 30, 36, 38, 40). These Prox-1 positive vessels were found surrounding the base of the aorta and pulmonary trunk and running along their length. We also noted that these vessels had clusters of cells at regular intervals that may be involved in the formation of the lymphatic valves. Thus, the avian embryonic heart appears to acquire a lymphatic network during cardiogenesis in parallel with blood vessel development. In addition to Prox-1 expression in these vessels, we found Prox-1 in cells on the downstream surfaces of the cardiac valves and valves of the great vessels (stage 38 and 40) and in cells within the myocardial ventricular wall (as early as stage 17). At stages when the heart has just looped and has distinct atrial chambers, Prox-1 labeled cells are scattered over the surface of the atrium, the ventricle, and the base of the outflow tract. Thus Prox-1 could have roles beyond lymphangiogenesis in early cardiac development prior to the appearance of definitive blood vessels by contributing to cardiac valve development and to cardiac myocardial development. Source of Research Support: NIH R01 ES013507, HL65314, HL0775436, HL083048, NASA Glenn IR&D 04-54.

81. Establishment of the Cardiovascular Lineages from Flk1+ Multipotential Cardiovascular Progenitor Cells

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In the mouse embryo the heart primordium is established from mesodermal cells that migrate from the primitive streak (PS) to the anterior-proximal side of the embryo and contribute to myocardium and endothelial cells of the endocardium. Lineage tracing studies have demonstrated that the cardiac lineage arises from a population that expresses the VEGF receptor Flk1, suggesting that it may develop from a progenitor with vascular potential. To identify and characterize the earliest stages of cardiac development, we have used the ES cell differentiation model, with ES cells carrying the GFP cDNA targeted to the locus of the PS gene brachyury (GFP-Bry cells). Analysis of early stage serum-induced embryoid bodies (EBs) revealed the presence of three distinct populations based on expression of GFP and Flk1: GFP-Bryneg/Flk1neg GFP-Bry+/Flk1neg and GFP-Bry+/Flk1+. Evaluation of the developmental potential of these populations revealed that hemangioblasts defined as blast colony-forming cells (BL-CFCs) were found in the GFP-Bry+/Flk1+ fraction whereas cells with cardiac potential segregated to the GFP-Bry+/Flk1neg population. When allowed to aggregate for 24 hours the GFP-Bry+/Flk1neg population generated a second Flk1+ population (2o Flk1) that contained all cardiomyocyte potential and preceded expression of the earliest know cardiac transcription factors. To determine the lineage potential of the cells that generated cardiomyocytes, cells from the 2o Flk-1 population were cultured in methylcellulose in the presence of factors known to function in the heart primordium. Following 4 to 6 days of culture, distinct colonies of cells developed that displayed cardiac, endothelial and VSM potential. Single cell deposition experiments revealed that the colonies were clonal indicating

that they arise from a cardiovascular colony-forming cell (CV-CFC). Analysis of head-fold stage embryos demonstrated the presence of a similar Flk1+ CV-CFC. Together, these findings demonstrate the existence of a Flk1+ multipotential cardiovascular progenitor that develops following the establishment of the hemangioblast lineage.

82. Versican proteolysis mediates myocardial regression during outflow tract development
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An important phase of cardiac outflow tract (OFT) formation is the remodeling of the distal region of the common outlet in which the myocardial sleeve is replaced by with smooth muscle. We find that the expression of the proteoglycan versican is reduced prior to the loss of myocardium from the distal cardiac outlet concomitant with an increase in production of the N-terminal cleavage fragment of versican. To test whether versican proteolysis plays a role in OFT remodeling, we determined the effects of adenoviral-mediated expression of a versican variant, V3, devoid of known matrix metalloproteinase cleavage sites and an N-terminal fragment of versican (G1). V3 expression promoted an increase in thickness of the proximal OFT myocardial layer independent of proliferation. In contrast, the G1 domain caused thinning and interruptions of the OFT myocardium. These in vivo findings were consistent with findings using cultured primary cardiomyocytes showing that the V3 promoted myocardial cell-cell association while the G1 domain caused a loss of myocardial cell-cell association. Taken together, we conclude that intact versican and G1-containing versican cleavage products have opposing effects on myocardial cells and that versican proteolysis may facilitate the loss of distal myocardium during OFT remodeling.

83. LRRC10 is essential for cardiac development and function during early development
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The molecular events that lead to normal cardiac development and function remain largely unknown. We identified Leucine Rich Repeat Containing protein 10 (LRRC10) as a cardiac-specific factor by employing an in silico approach followed by in situ hybridization and Northern blot analyses. Mouse LRRC10 expression was markedly elevated upon birth, and a high level of expression was maintained in the adult heart. LRRC10 exhibited dynamic intracellular localization patterns in cardiomyocytes. Cardiomyocytes from embryos and newborns showed diffuse cytoplasmic and nuclear staining of LRRC10. In contrast, striking striations were observed in adult cardiomyocytes, colocalizing with the Z-line markers. Electron micrographs showed that LRRC10 was localized in the diad region where the sarcoplasmic reticulum interacts with the transverse tubule, which is located along the Z-line. To investigate the developmental roles of LRRC10 in the heart, we performed morpholino experiments using zebrafish embryos. Knockdown of Lrrc10 in zebrafish embryos (morphants) resulted in severe cardiac morphogenic defects including a cardiac looping failure accompanied by a large pericardial edema. The Lrrc10 morphants also exhibited functional defects as evidenced by a decrease in ejection fraction and cardiac output, which likely led to embryonic lethality between day 6 and 7 post fertilization. Further investigations into the underlying mechanisms of the cardiac defects revealed that the number of cardiomyocytes was reduced in the morphants. Expression of cardiac genes was deregulated in the morphants including an increase in anf, and a decrease in cmlc2. To assess the molecular function of LRRC10, we set out to identify cofactor(s) of LRRC10 by yeast two-hybrid and proteomics approaches. We are currently investigating physical and functional interaction between LRRC10 and its cofactor. Taken together, the present study demonstrates the critical functions of LRRC10 in cardiac development and function, which will enhance our understanding of congenital heart defects and heart disease.

84. Hesr1 and Hesr2 regulate atrioventricular boundary formation in the developing heart through the repression of Tbx2

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The establishment of chamber specificity is an essential requirement for cardiac morphogenesis and function. Hesr1/Hey1 and Hesr2/Hey2 are specifically expressed in the atrium and ventricle, respectively, implicating these genes in chamber specification. In our current study, we show that the forced expression of Hesr1 or Hesr2 in the entire cardiac lineage results in the reduction or loss of the atrioventricular (AV) canal. In the Hesr1-misexpressed heart, the boundaries of the AV canal have a poorly defined, and the expression levels of specific markers of the AV myocardium, Bmp2 and Tbx2, are weakened or undetectable. More potent effects could be observed in Hesr2-misexpressed embryos, in which the AV canal appears to be absent entirely. These data suggest that Hesr1 and Hesr2 may prevent cells from expressing the AV canal specific genes that lead to the precise formation of the AV boundary. Our current findings further indicate the possibility that Tbx2 expression is directly suppressed by Hesr1 and Hesr2. Furthermore, we find that the expression of Hesr1 and Hesr2 is independent of Notch2 signaling. Taken together, our data thus demonstrate that Hesr1 and Hesr2 play crucial roles in AV boundary formation through suppression of Tbx2 expression.

85. Calcium Manipulation in Zebrafish Embryos Affects Left-Right Asymmetry of the Heart and Brain

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Background: Vertebrates develop left-right asymmetries that include a left-sided heart, and specialized areas in each side of the brain. Calcium signals are believed to play a role in the specification of left-right asymmetry, by translating cilia-dependent fluid flow into asymmetric patterns of gene expression. Objective: The objective of this study is to determine the role of early calcium signals on the left-right asymmetry of the zebrafish heart and brain. Design/Methods: Calcium signals were manipulated in zebrafish embryos using thapsigargin, an inhibitor of the endoplasmic reticulum (ER) calcium pump. The embryos were treated with 0.5 M thapsigargin during early gastrulation (4-6 hpf), mid-gastrulation (6-8 hpf), late gastrulation (8-10 hpf) or early somitic stages (10-12 hpf). The phenotype was analyzed at the desired stage of development with subtractive imaging to determine the orientation of the heart, immunolabelling to visualize cilia and mRNA in situ hybridization to examine gene expression pattern. Results: At 30 hpf, the heart was centralized or reversed in 53% of the thapsigargin-treated embryos (4-6 hpf) (n=72) vs 5% of the DMSO-treated control embryos (n=75). The embryos were most sensitive to thapsigargin during early and mid-gastrulation with subsequent decrease in heart laterality defects. Kupffer s vesicle formation at 12-13 hpf was affected in 73% of the thapsigargin-treated embryos, while only 2% of the control embryos displayed abnormalities of this structure ($p<0.01$). The absence of Kupffer s vesicle correlated with increased incidence of heart laterality defects. Left-right patterning in the brain was examined by pitx2α in situ hubridization and was found to be reversed or bilateral in 56% of the early treated embryos (n=97) vs in only 10% of the control embryos ($p<0.05$). Conclusions: Inhibition of the ER calcium pump by thapsigargin during gastrulation inhibits development of the Kupffer s vesicle and causes laterality defects in the zebrafish heart and brain. These results suggest an additional role of calcium in left-right asymmetry well before its previously recognized role in sensing fluid flow in the Kupffer s vesicle.

86. Epithelial lineage-specific expression of the Noonan-syndrome mutation Q79R-Shp2
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Noonan Syndrome (NS) is the most common non-chromosomal genetic disorder associated with cardiovascular birth defects, occurring in up to 1:1200 live births. NS is characterized by short stature, facial dysmorphia, skeletal anomalies, and congenital heart disease. Among the heart defects, pulmonary valve stenosis and hypertrophic cardiomyopathy are most prominent. Gain-of-function mutations in the protein tyrosine phosphatase Shp2 have been identified in 50% of NS families. Shp2 is known to regulate numerous cellular events including proliferation, differentiation, and migration. Our aim is to understand the exact cellular mechanism(s) by which dysfunction of Shp2 can cause valve malformation. Using a Tie2Cre/loxP-based transgenic approach, we have created a mouse model for NS in which Shp2 containing the NS mutation Q79R is selectively overexpressed in the epithelial cell lineage, in particular in the mesenchyme of the developing endocardial cushions. In the resulting embryos, immunohistochemistry demonstrated strong expression of mutant Shp2 in the developing cushions as well as in the endocardium and vasculature. However, Q79R-Shp2 expression was embryonic lethal by 14.5 dpc. Dying embryos displayed nuchal edema, indicating that heart failure may be at least in part responsible. Embryos were taken at 13.5 dpc for detailed histological examination of the hearts. At this time, the number of live embryos found was slightly below expected Mendelian ratios. Cardiac defects such as enlarged atrioventricular endocardial cushion primordia and ventricular non-compaction were found. However, penetrance of the cardiac phenotype was incomplete, suggesting that extracardiac effects of mutant Shp2 expression contributed to the embryonic lethality. Expression of mutant Shp2 was associated with increased p44/42 MAPK activation in a subset of cells within the endocardial cushions, suggesting that hyperactivation of this signaling pathway may be part of the downstream mechanism.

87. Coronary artery development during late embryonic and postnatal stages
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The progenitor cells of the coronary arteries originate from the proepicardium, which forms the epicardium by E11.0 and a capillary plexus connected to the aorta by E13.5. Little is known on the subsequent maturation of the coronary arteries. We hypothesized that coronary artery maturation is a highly-organized process, which continues after birth to adjust to the postnatal increase in vascular pressure and postnatal maturation of the heart. Hearts from embryonic (E15.5, E18.5), neonatal (N1.5, 1w, 2w), and adult (8 w) FVB mice were analyzed by immunofluorescence using markers for endothelial cells (lectin, VWF and PECAM), vascular smooth muscle cells (VSMCs) (alpha-smooth muscle actin), fibroblasts (vimentin), and cardiac myocytes (MF20). At E15.5, coronary vessels consisted of one layer of endothelial cells, surrounded by cardiomyocytes. Few squamous SMCs were observed between the endothelium and myocardium. At E18.5, coronary vessels consisted of two layers with a continuous layer of round-shaped VSMCs surrounding the endothelial tubes. Perivascular fibroblasts were sparse around the vessels at E18.5, and became more abundant during subsequent development. Individual extracellular matrix (ECM) components were also analyzed. At E15.5, collagen IV-VI, laminin and fibronectin were present at the basement membrane of the endothelial cells (basal lamina). At E18.5, these ECM components, together with collagen I, were also observed surrounding the VSMCs. From N1.5 onwards, progressive accumulation and patterning of the ECM components took place. Two weeks after birth, the resulting pattern consisted of fibronectin in the basal lamina, laminin and collagen IV in the basal and external lamina, collagen I, III and V in the external lamina and in the extracellular space between the perivascular fibroblasts. Collagen VI was present in all three regions. In conclusion, coronary artery formation includes the progressive assembly of its different components before birth and extensive remodeling of the ECM after birth.

88. SHP-2 is Required for the Maintenance of Cardiac Progenitors

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The isolation and culturing of cardiac progenitor cells has demonstrated that growth factor signaling is required to maintain cardiac cell survival and proliferation. In this study, we demonstrate that SHP-2 activity is required for the maintenance of cardiac precursors in vivo. In the absence of SHP-2 signaling cardiac progenitor cells downregulate genes associated with early heart development and fail to initiate cardiac differentiation. We further show that this requirement for SHP-2 is restricted to cardiac precursor cells undergoing active proliferation. By demonstrating that SHP-2 is phosphorylated on Y542/Y580 and that it binds to FRS-2, we place SHP-2 in the FGF pathway during early embryonic heart development. Furthermore, we demonstrate that inhibition of FGF signaling mimics the cellular and biochemical effects of SHP-2 and that these effects can be rescued by constitutive active/Noonan syndrome associated forms of SHP-2. Collectively, these results show that SHP-2 functions within the FGF/MAPK pathway to maintain survival of proliferating populations of cardiac progenitor cells and further suggest that the FGF/SHP-2/MAPK pathway may represent a general pathway required to maintain proliferating progenitor populations.

89. Global gene expression analysis of zebrafish heart regeneration

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Mammalian hearts cannot regenerate in response to tissue damage. In contrast, zebrafish hearts regenerate even when up to 20% of the ventricle is amputated. The mechanism of zebrafish regeneration is not understood. To systematically characterize this process at the molecular level, we generated transcriptional profiles of zebrafish cardiac regeneration by microarray analyses. Distinct gene clusters were identified based on temporal expression patterns of early stage of heart regeneration. Genes coding for wound response/ inflammatory factors, secreted molecules and matrix metalloproteinases are expressed in regenerating heart in sequential patterns. Comparisons of gene expression profiles between heart and fin regeneration revealed a set of regeneration core molecules as well as tissue specific factors. The expression patterns of several secreted molecules around the wound suggest that they play important role in heart regeneration. We found that both Platelet-derived growth factor-a and -b (pdgf-a and pdgf-b) are upregulated in regenerating zebrafish hearts. To study the functions of these differentially expressed genes at cellular level, we established primary culture of adult zebrafish cardiomyocytes. The majority of the cardiomyocytes are mononucleated. Cardiomyocytes in our primary culture were positively identified by staining with antibodies against the cardiomyocyte markers tropomyosin and MEF2. We observed that PDGF-B homodimers induce increased DNA synthesis in adult zebrafish cardiomyocytes. In addition, we demonstrate that a chemical inhibitor of PDGF receptor decreases DNA synthesis of cardiomyocytes in vivo during regeneration. Our data indicate that zebrafish heart regeneration is associated with sequentially upregulated wound healing genes and growth factors, and suggest that PDGF signaling is required.

90. Sox9 is required during early and late stages of heart valve development

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Heart valve structures derived from mesenchymal cells of the endocardial cushions (EC) are composed of highly organized cell lineages and extracellular matrix (ECM). However the molecular mechanisms required for lineage determination during valve development and adult valve maintenance are not well defined. Recent studies have identified shared regulatory pathways of valve formation with cartilage and tendon development. Sox9 is a transcription factor required for both early and late stages of cartilage formation that is also expressed in the developing valves of the heart. The requirements for Sox9 function during valvulogenesis and adult valve homeostasis in vivo were examined by conditional inactivation of Sox9 using Tie2-cre and Col2a1-cre transgenes. Using Tie2-cre, sox9 function was reduced in valve precursor cells of the ECs and consequently all endothelially-derived valve structures during early stages of valvulogenesis. In contrast, Col2a1-cre recombines with sox9 in a subset of differentiating cell types that lie along the edge of heart valve leaflets during later stages of valve development. Sox9flox/flox;Tie2-cre mice die before E14.5, and although EC formation is initiated, cushions are hypoplastic with reduced cell proliferation and altered ECM organization. Sox9flox/flox;Col2a1-cre mice die at birth with thickened heart valve leaflets that display reduced expression of sox9-regulated cartilage proteins including type II collagen and cartilage link protein. Consequently, overall ECM organization is disrupted in heart valves of Sox9flox/flox;Col2a1-cre mice. Thickened valve leaflets and calcium deposits, characteristic of pathological valve disease, are observed in heterozygous adult Sox9flox/+;Col2a1-cre mice, consistent with a potential role for Sox9 in preventing tissue mineralization. These studies demonstrate that Sox9 is required early in valve development for expansion of the precursor cell population and later is required for normal expression and distribution of valvular ECM proteins. Further, this study identifies a potential role for Sox9 in valve disease mechanisms associated with alterations in ECM homeostasis.

91. Hypoxia-inducible transcription factor-1a plays a protective role in outflow tract remodeling

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Our previous studies demonstrated that cardiomyocyte hypoxia and apoptosis are essential for developmental remodeling of the avian outflow tract (OFT) in the transition to a dual circulation. Accumulation of the HIF-1a protein mediates transcriptional responses to hypoxia. However, whether or not HIF-1a induced by hypoxia mediates the cardiomyocyte apoptosis and therefore influences the remodeling of OFT remains uncertain. To answer this question, constitutively active HIF-1a in adenovirus vector (generously supplied by G. Semenza) was used to transduce chicken hearts in ovo at stage 17-18; apoptosis and heart morphology were evaluated at subsequent stages of OFT remodeling. 13 of 17 and 12 of 18 of HIF-1a over expressing hearts showed significantly less LysoTrack Red (LTR) positive cells in myocardium at stage 25 and 30, respectively, compared to control group. Similarly, the protein level of active Caspase 3 in myocardium detected by immunohistochemistry was significantly reduced due to over expression of HIF-1a. In 13 out of 19 embryos, over expression of HIF-1a resulted in morphological defects including double outlet right ventricle, ventricular septal defect, and excessive myocardium in OFT cushion at stage 34-35. The data suggest that HIF-1a may play a protective role for cardiomyocytes by reducing cell death in the OFT remodeling. We are also functionally testing the downstream targets of HIF-1a in this model. Blockade of endogenous VEGF by in ovo injection of adenovirus expressing VEGF Trap caused heart defects in 19 out of 20 embryos. The abnormalities included conotruncal defects, proliferative cushion mesenchymal cells with poorly developed valves, VSD and thin ventricular wall. Nearly all of the VEGF Trap-treated embryos had increased numbers of LTR positive cells in myocardium at stage 25 and 30. Our data suggest that hypoxia induced HIF-1a and downstream VEGF in OFT myocardium serve to prevent cardiomyocytes from apoptosis in OFT remodeling. Supported by NIH grant HL65314.

92. Tip60 gene knockout causes embryolethality, and haploinsufficiency revealed by stress-induced alteration of myocardial cell kinetics

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Tat-interactive protein 60 (Tip60) is a member of the MYST family, proteins in which contain atypical histone acetyltransferase (HAT), Zinc-finger and chromo-domains. Because several functions have been attributed to Tip60, including the regulation of DNA transcription, repair and apoptosis, its function in the cell has been unclear. Findings during the past year have revealed that Tip60 (i) is one of six "hub" proteins which modulates effects of gene mutation, (ii) directly acetylates p53 to enable its pro-apoptotic activity and (iii) is an intermediate in p38 MAP kinase signaling which induces senescence in cells including cardiomyocytes. Pursuant to our finding that Tip60 is expressed in the embryonic and adult heart, we globally deleted the Tip60 gene (Htatip) from the mouse, revealing that homozygous ablation causes embryolethality before the blastocyst stage of development. Although heterozygous adult tissues contain haploinsufficient levels of Tip60 mRNA, Tip60 protein is maintained at normal levels, explaining the absence of a heterozygous phenotype. To assess whether cardiac stress induces a haploinsufficient phenotype in Tip60+/- hearts, aortic-banding revealed that hypertrophied Tip60+/- myocardium contains significantly increased numbers of cycling as well as mitotic cells, some of which appear to be cardiomyocytes, concomitant with reduced apoptosis, in comparison with Tip60+/+ controls. These findings indicate that Tip60 is a vital molecule which regulates cellular kinetics in the myocardium.

93. Topogenesis of the pulmonary vein orifice in Xenopus laevis embryos.

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The topogenesis of the orifice of the common pulmonary vein (OCPV) is still not well understood. Current matters of dispute are, firstly, the question as to whether the future OCPV is primarily connected to the primitive embryonic atrium or to the sinus venosus and, secondly, the question as to whether the OCPV normally arises either from a solitary anlage that is primarily located at the embryonic midline or from the left-sided portion of a primarily paired anlage. We studied the development of the OCPV in Xenopus laevis embryos by scanning electron microscopy (stages 39 to 46). We have found that the OCPV appears during stage 41. From the time point of its first appearance onward it is located, firstly, to the left of the embryonic midline and, secondly, cranial to a circular fold that demarcates the border between the confluence of the systemic veins (sinus venosus) and the primitive embryonic atrium. Our findings are in accord with the view that the OCPV is primarily connected to the left atrium and with previous data from chick and reptilian embryos suggesting that the OCPV normally arises from the left-sided portion of a primarily paired anlage.

94. The Cardiac Crescent Exhibits Nkx2.5 Dependent Left-Right Asymmetry

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Previous studies have defined a role for Nodal, Cripto and Pitx2c in left-right (LR) asymmetry. The transcriptional patterning of the cardiac crescent that is populated by cardiac precursor cells has not been previously described. We have engineered and utilized a 6 kb Nkx2-5-EYFP transgenic mouse that directs reporter expression in cardiac progenitors and combinatorially mated them into the wildtype (WT) or Nkx2-5 mutant backgrounds. Staged embryos were bisected, cardiac progenitors were collected using FACS and the signature of gene expression of cardiac progenitors that populate the left vs. the right regions of the cardiac crescent were examined. We observed that the left side of the WT cardiac crescent had increased expression of Nodal, Cripto and Pitx2 transcripts that are known to have functions critical

to future chamber myocardium as well as Nkx2-5. The right side of the crescent was not enriched for a specific gene program. We analyzed the same LR transcriptional program in the Nkx2-5 mutant cardiac crescent. Nkx2-5 nulls exhibited a loss of the left-sided enrichment for Nodal, Cripto and Pitx2c and the cardiac transcripts that were enriched on the left side of the WT crescent revealed equal expression in the Nkx2-5 null crescents. In contrast, many right-side enriched transcripts retained the patterns they exhibited in the WT. Since the left-sided program was enriched in genes predicted to function in ventricular chamber myocardium, which appears to be defective in the Nkx2.5 nulls, the disruption of this program in the nulls supports a functional role for early LR asymmetry in patterning future myocardial lineages. Analysis of Nodal and Cripto promoters revealed the presence of evolutionary conserved Nkx2-5 binding sites (NKE). We utilized EMSA, mutagenesis, supershift, ChIP assays, luciferase transcriptional assays and a Nkx2-5 inducible ES/EB system to verify that Nodal and Cripto are direct Nkx2-5 downstream targets. Our data support the hypothesis that Nkx2-5 functions in the cardiac progenitor cell population to maintain the left-right patterning of the cardiac crescent.

95. Zebrafish Atrio-Ventricular Valve Development and Early Maturation Occurs Between 6dpf and 16dpf

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The zebrafish heart begins to contract rhythmically by 22hpf and the first signs of a differentiated atrium and ventricle appear by 37hpf (Walsh and Stanier, 2001). By 45hpf, the developing zebrafish heart begins to function by preventing retrograde blood flow (Stanier, 2005). The cardiac cushions, located at the atrio-ventricular boundary, are formed by 96hpf, while valve leaflets appear at 7dpf (Stainier, 2005). Our current studies focus on the stage following AV cushion formation, which involves valve elongation and the beginning of valve maturation. We utilized the Tie-2:GFP expressing line in order to elucidate what cell types are present during valve elongation as well as how the valves achieve elongation. Preliminary results indicate the AV valves elongate both by cell migration and proliferation. Through a series of BrdU uptake experiments, histochemical analysis, and immunohistochemistry, we found that the AV valves proliferate at 4-6dpf and 14-16dpf, but not between 6-14dpf or after 16dpf. Early proliferation happens in the Tie-2 positive cells while Tie-2 negative cells are proliferating later. It is between 8dpf and 14dpf that the valves increase in length without any evidence of proliferation. During this period of non-proliferation, there are punctate focal complexes in AV valve cells while at 16dpf, when proliferation is slowing, large focal adhesions appear to surround the entire cell body. After 16dpf, the beginning of valve maturation takes place as revealed by the deposition of both collagen and versican along the length of the zebrafish AV valve. These data suggest that zebrafish AV valves begin elongating after 6dpf by the processes of cell migration and proliferation while setting the stage for final elongation and maturation starting with 16dpf.

96. The role of the Sodium Calcium Exchange (NCX-1) on the electrical activity of the early stages of chick heart development

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The heart is the first functional organ in the developing embryo and the major functional characteristic of the cardiomyocyte are their contractile and electrical properties. The time course of the development of electrical properties in the cardiomyocyte during early heart development remains inadequately defined. The development of cardiac electrophysiology is significant for normal heart development, as well as for understanding the possible later development of pathologies in the adult myocardium. To date, the primary focus of effort in early heart development and cardiac cell differentiation has been on the identification of regulatory genes and growth factor that control development. Our experiments indicate that maintenance of specific intracellular ionic levels including calcium are critical during the early stages of cardiac differentiation. However, the potential contribution of calcium signaling remains largely unexplored during early stages of heart development. In mature myocardiocytes, the sarcolemmal

Na+/Ca2+ exchanger (NCX) rapidly transport Ca2+ during excitation-contraction coupling and is the dominant myocardial Ca2+ efflux mechanism. The NCX is bidirectional transporter that catalyzes the exchange of Na+ for Ca2+, depending of the electrochemical gradients of the substrate ions. Currently 3 NCX genes have been identified; NCX1 is expressed in various organs including the heart. It has been shown that NCX play a important role in excitation-contraction coupling and Ca2+ homeostasis in the heart. Also it has been shown that a rapid up regulation of NCX1 mRNA occurs in the heart as response to overload. Furthermore the NCX is also upregulated in end-stage heart failure conditions. Variations in concentration of NCX1 protein among myocytes form failing human heart are inversely related to variation in the frequency-dependent increase in diastolic calcium, which results in diastolic dysfunction. Thus we are interested in elucidate at which stage of development the NCX1 is present in the different regions of the heart, and which is it role in the modulation of the electrical activity as well to the intracellular calcium levels during the different stages of development. Our data indicate that NCX1-mRNA is equally expressed in atria and ventricle at early stages of development (St 13) but is predominantly express in the ventricle at latter stages of development (St 40). Blocking of the NCX1 with 10 μM KB-R7943 induces an increase in the duration of the action potential in stages 25 to 29. These changes are characterized by an increase of AP amplitude, decrease in rise time and increase in the net are under the AP, and a net decrease in the maximal rate of rise. Thus these results suggest that NCX1 plays an important role in Ca2+ homeostasis in early stages as St-25. Supported by LPP-AHA-SDG 0530140N

97. Pitx2c and Nkx2-5 are required for the formation and identity of the pulmonary myocardium

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The pulmonary vein is sleeved by myocardium, which is a major source of atrial fibrillation, and is involved in congenital sinus venosus defects. Little is known about the cellular origin and mechanism of formation of the pulmonary myocardium. By genetically labeling atrial cells and their daughters, we show that, contrary to current belief, the pulmonary myocardium is not derived from the atrial working myocardium. The development of the pulmonary myocardium can be divided into two phases. First, a myocardial population forms at the connection of the pulmonary vein and the atrium, either by differentiation of mesenchymal precursor cells around the pulmonary vein into myocardium, or by in-growth of the neighboring mediastinal myocardial cells into the mesenchyme. Secondly, shortly after these first pulmonary myocardial cells have been formed, they initiate a phase of rapid proliferation to form the pulmonary myocardial sleeve. Pitx2c-deficient embryos develop a pulmonary vein. However, they fail to form the pulmonary myocardial sleeve. Closer examination revealed that the first pulmonary myocardial cells do not form in the absence of Pitx2c, and that the phase of rapid proliferation is not initiated in the cells around the pulmonary vein. Further labeling analyses demonstrated that whereas the systemic venous return derives from Nkx2-5-negative precursor cells, the pulmonary myocardium derives from Nkx2-5-expressing cells, providing additional evidence for a distinct origin of the two venous systems. Nkx2-5 and its target gap-junction gene Cx40 are expressed in the atria and pulmonary myocardium, but not in the systemic venous return, which expresses the essential pacemaker channel Hcn4. When Nkx2-5 doses are lowered, the pulmonary myocardium switches to a Cx40-negative, Hcn4-positive phenotype resembling that of the systemic venous return. In conclusion, our results provide evidence for key-roles of Pitx2c and Nkx2-5 in the formation and identity of the pulmonary myocardium.

98. Modest maternal caffeine exposure affects developing embryonic cardiovascular function and growth

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Caffeine consumption during pregnancy reportedly increases the risk of spontaneous abortion and fetal growth restriction. However, the impact of modest maternal caffeine ingestion upon the developing embryonic cardiovascular (CV) function and growth remains unknown. Caffeine (10 mg/kg subcutaneous) was administered daily to pregnant CD-1 mice from embryonic days (EDs) 9.5 to 18.5 of a 21 day gestation. Maternal and embryonic CV function was assessed before (baseline) and 30 minutes after caffeine administration (peak maternal caffeine concentration) using high frequency echocardiography at EDs 9.5, 11.5, 13.5, and 18.5. Caffeine did not influence maternal CV function nor influence embryo resorption rate. However, the ED18.5 embryonic crown-rump length and body weight were decreased and the placenta weight increased. While caffeine did not influence baseline embryonic hemodynamics until ED13.5 the caffeine increased the embryonic carotid artery flow and decreased embryonic aortic flow at ED 18.5. At peak maternal caffeine concentration, ED 11.5 embryonic carotid artery, aorta, and umbilical artery flows were decreased from baseline. The embryonic carotid artery flow at peak maternal caffeine concentration remained reduced at EDs 13.5 and 18.5 while aortic and umbilical artery flows normalized. Maternal treatment with the adenosine A2A receptor inhibitor reproduced the embryonic hemodynamic effects of maternal caffeine exposure. Adenosine A2A receptor gene expression of ED11.5 embryo and ED18.5 uterus were down-regulated. Our results suggest that modest maternal caffeine exposure has adverse effects on developing embryonic CV function and growth, possibly mediated via adenosine A2A receptor blockade.

99. Tbx5 Patterns Boundaries in the Developing Heart

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Heart chamber identity develops after cardiogenic initiation, suggesting that chamber patterning must be determined subsequently. Based on its early antero-posterior cardiac expression, the T-box transcription factor Tbx5 is an excellent candidate for a molecule that might be responsible for cardiac chamber patterning. We have performed regulated spatiotemporal deletions of Tbx5 in the mouse to examine the role of Tbx5 in chamber patterning. We demonstrate an uncoupling of the role for Tbx5 in early cardiac differentiation from later fine morphogenesis of distinct cardiac structures. These studies uncovered a critical requirement for Tbx5 in the formation of the interventricular septum (IVS) between E9-10, which was further investigated by deleting Tbx5 in subdomains of the developing ventricles. We demonstrate that Tbx5 is necessary to specify ventricular chamber identity and is required specifically at the interventricular midpoint for initiating IVS outgrowth and development of the distal conduction system. The defects caused by the ventricular disruption of Tbx5 underscore its importance in establishing chamber boundary element identity and show a direct role for Tbx5 in positioning and outgrowth of the IVS. The studies presented here extend the understanding of the role of Tbx5 in cardiogenesis, arguing that congenital heart defects may be influenced by local spatiotemporal changes within chamber precursors.

100. miR-138 Is Necessary for Cardiac Patterning in Zebrafish

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MicroRNAs (miRNAs) are small non-coding RNAs that inhibit protein expression by RNA degradation and translational inhibition. Many miRNAs are expressed in tissue-specific patterns, and may regulate developmental processes, but the functions of most remain unknown. miR-138 is expressed in the zebrafish heart and central nervous system (CNS), as well as the mouse CNS. We found that morpholino knockdown of miR-138 in the zebrafish resulted in cardiac dysfunction by 48 hours post fertilization. Defects in myocardial cell shape were observed that resulted in a lack of cellular elongation typically seen in the ventricle. In many cases, miR-138 was required for looping of the heart tube and development of the ventricular chamber. Molecular analyses revealed expansion of some atrioventricular canal (AVC) markers into the ventricular region in morpholino injected embryos, suggesting a dysregulation of cardiac patterning. In vitro and in vivo, we show that Versican, which is usually expressed in the AVC, is a direct target of miR-138. These results suggest that mir-138 regulates the patterning and morphogenesis of the zebrafish heart, in part through the regulation of Versican.

101. Touch the embryonic heart: Enlarged cast models of chick hearts at looping stages

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We present, to our best knowledge, the first touchable casts (RepliCasts) of embryonic chick hearts at looping stages in sham-operated and cardiac neural crest (CNC) ablated embryos on basis of magnetic resonance microscopy (MRM). Three-dimensional (3D) reconstructions of these datasets were already shown in 2004 (1). Unlike in these 3D reconstructions, on RepliCasts fewer structures were virtually cut off for optimal stabilisation of the model. In comparison to 3D reconstructions, RepliCasts show higher contrast and clearer spatial information about the size differences or about spatial relations of important structures like, for example, the aortic arch arteries. Additionally, tactile sensations provided by RepliCasts are able to highly improve the spatial sense of the complex morphological structures of embryonic hearts. For embryonic chick specimen preparation a dual-contrast method was used that combines perfusion fixation and immersion in fixative with a macro-molecular gadolinium-based contrast agent. MRM was performed on a 9.4-T magnet using custom-designed radiofrequency coils (Helmholz pair and solenoid coils), resulting in image resolutions of 25-µm3 isotropic voxels for stage 22 [day 4] chick embryos. After segmentation with automatic interpolation on original MRM data sets by software developed at our institution endocardial und endoluminal borders were generated by interactively choosing the grey scale threshold. Virtual surface models were created including only connected structures and were then enlarged by the factor 100 for all three dimensions resulting in a total magnification factor of one million. The results were controlled using red-/blue glasses before 3D printing by the inkjet technique (ZPrinter 310, ZCorporation, Burlington, USA). Magnified 3D models of embryonic chick hearts are expected to highly simplify the understanding and teaching of normal and abnormal heart development. Reference: (1) Yelbuz et al., 2004. Circulation. 2004;108;e154-e155.

102. Requirement of Notch signaling in early vertebrate cardiogeneis
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During early vertebrate embryogenesis, the Nkx2.5–expressing heart field is segregated into two distinct cell populations. Studies in mouse and chick have shown that the primary population gives rise to the myocardial heart tube that eventually becomes the atria and ventricles , while the secondary population gives rise to the outflow tract. Understanding how and when these two populations are established, and how their differentiation is coordinated, is fundamental to understanding heart morphogenesis. I have chosen to analyze the involvement of Notch signaling in these processes using the zebrafish model. Analysis of the Notch signaling null mutant, mindbomb, and disruption of candidate Notch pathway genes zfjagged3 and zfnotch6 demonstrate changes in myocardial cell number and disrupted outflow tract development that may provide insight into the formation of heart defects present in sufferers of Alagille syndrome, the result of reduced Notch signaling.

103. The Role of Mechanical Feedback in the Regulation of Cardiac C-Looping
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Cardiogenesis involves a carefully coordinated series of morphogenetic events, but how these events are controlled is poorly understood. Some researchers espouse the view that cells simply follow instructions provided by successive changes in gene expression. Others believe that biological form evolves from a dynamic interaction between genetic and environmental factors (chemical and mechanical), with adjustments being made continually through feedback mechanisms. In this study, we explore the role of mechanical feedback in the regulation of cardiac c-looping, as the relatively straight heart tube bends and twists into a c-shaped tube with outer (convex) curvature normally directed toward the right side of the embryo. Embryonic chick hearts at HH stages 10-12 were subjected to mechanical interventions that perturb normal looping (e.g., removal of splanchnopleure, right or left primitive atrium, or both atria), and these same interventions were simulated in two- and three-dimensional computational models for the looping heart. The models, which are based on the fundamental principles of mechanics, include stress-regulated growth and cytoskeletal contraction. Model predictions generally agree well with experimental observations of looping morphology and quantitative measurements of morphogenetic strains. For example, cutting the left atrium at stage 10 leads to left looping in both the model and experiments. These results suggest that cardiac morphogenesis consists of a series of self-regulated processes initiated by mechanical perturbations. These perturbations may be caused by genetic activity, which also sets system parameters.

104. Disruption of Smad4 in neural crest cells leads to mid-gestation death with
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Neural crest cells (NCCs) are a group multipotent, migratory cells that are generated from the interface of dorsal ectoderm and neural tube along the neuraxis, and play critical roles during craniofacial and cardiovascular development. Previous studies have demonstrated that Tgf-β/Bmp signaling pathways are essential for normal development and functions of NCCs. Smad4 encodes the only common Smad protein in mammals, which is a critical nuclear mediator of Bmp/Tgf-β signaling. In this work, we sought to investigate the roles of Smad4 for development of NCCs. To overcome the early embryonic lethality of Smad4 null mice, we specifically disrupted Smad4 in NCCs using a Cre/loxP system. The mutant mice (Wnt1cre; Smad4loxp/loxp) died at mid-gestation with defects in the facial primordia, pharyngeal arches, the outflow tract (OFT) and ventricles. The distal portion of OFT cushions in mutant embryos were hypoplasia, while the proximal OFT cushions appeared normal. The ventricle wall was also thinner than controls. Although many neural crest markers were expressed normally, decreased expression of Msx1 and Msx2 was apparent at E9.0~9.5. Expression of NCC migration markers were apparently reduced in

mutant embryos. The migrated NCCs exhibited increased cell death in the facial primordia and pharyngeal arches. The cell proliferation rate in these areas was not substantially altered. Expression of a set of genes involved in epithelial-mesenchymal interactions in facial primordia and pharyngeal arches was obviously down-regulated in mutant embryos. Taken together, we conclude that Smad4 mediated activities of Tgf□/Bmp signals are essential for appropriate NCC development.

105. In vivo and 3-dimensional Visualization of Coronary Artery Development by High-Resolution Optical Coherence Tomography (OCT)

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The establishment of a functioning CA system is one of the most critical but poorly understood processes during cardiovascular development. Due to the lack of suitable imaging technologies and methodological limitations, it is currently impossible to visualize this complex dynamic process on human or animal embryos in vivo. This study was conducted to visualize coronary artery (CA) development in vivo in embryonic hearts by use of high-resolution Optical Coherence Tomography (OCT). Embryonic chick hearts were studied at three critical stages of CA development. The OCT system used in this study was optimized for in vivo chick heart visualization and enables OCT movie recording with 8 frames/s, full automatic 3-dimensional (3D) OCT scanning, and blood flow visualization, i.e., Doppler OCT imaging. We present the very first in vivo recordings of developing CAs obtained from the hearts of chick embryos at three critical stages of CA development: day 8 (no CA connection established), day 9 (established CA connection with the aorta) and day 10 (further remodeling of the established CAs). We show in vivo Color Doppler OCT recordings which demonstrate onset of first blood flow from the aorta into the CAs in day 9 hearts. Further, we demonstrate animated 3D reconstructions of CAs arising from the aorta created from 3D volume data set acquired by OCT. Our data show that high resolution OCT systems facilitate real time in vivo imaging of a critical process during heart development.

106. A peptide incorporating the ZO-1 binding domain of Cx43 improves recovery of cardiac function following injury

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Gap junctions (GJ) composed of Cx43 are essential for organized propagation of cardiac action potential. Disease-associated GJ remodeling has been linked to increased incidence of arrhythmia in humans. The mechanisms that regulate injury-associated disruption of GJ function are poorly understood. We have previously shown that dynamic changes in interaction between Cx43 and the actin-binding protein ZO-1 are an important determinant of GJ size and distribution in cardiomyocytes (Hunter et al., Mol Biol Cell, 2006). As part of this work we developed a membrane-permeant peptide incorporating the carboxy-terminal ZO-1 binding domain of Cx43, designed to competitively disrupt interaction between Cx43 and ZO-1. Subsequent investigations demonstrated that treatment of cutaneous wounds with the Cx43 peptide, in a pluronic gel, enhanced wound healing; decreasing inflammation and accelerating closure, as well as improving mechanical properties and decreasing granulation tissue deposition. Prompted by results in skin, we initiated studies of the effects of the peptide in cardiac wound healing. To achieve this we developed a novel epicardial cryoinjury model that enabled production of a discrete wound displaying minimal variation in size and geometry. Additionally, we re-formulated peptide delivery for cardiac application from pluronic gel to an adherent methyl cellulose patch to provide timed-localized release. In echocardiographic studies of cryoinjured hearts treated with the Cx43-based peptide exhibited

significantly improved ventricular function (as evidenced by reduced dilation) compared to a groups exposed to a control peptide. Interestingly, this change was most pronounced at the first week, though persisted throughout the 8-week time course of the experiment. Morphological and histological analysis of injured hearts are underway to determine structural correlates of the observed improvement in cardiac function. Our data suggest a role for Cx43 CT sequences in tissue injury response in both heart and skin and may point to novel treatments for cardiac disease.

107. Transgenic lineage mapping and genetic ablation of the cardiac neural crest cell lineages
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Cardiac neural crest (CNC) play an important role in cardiovascular development since derivatives contribute to the outflow tract (OFT) septum and pharyngeal arch arteries (AAs) that give rise to the great vessels exiting the heart. Mutations in Pax3 lead to persistent truncus arteriosus (PTA), interventricular septal defects (VSD) and abnormal pharyngeal AA remodeling. The type of cardiovascular defects present in these Pax3-deficient mice are similar to those observed in many cases of human congenital heart defects. Experiments in both mouse and chick have shown that Pax3-deficient CNC migrate abnormally and in reduced numbers. As a result, fewer cells are able to colonize the AAs and OFT, resulting in PTA and subsequent VSDs. However, the specific time-window required for Pax3 expression in the CNC lineage is still controversial. Furthermore, not much is known about the specific nature by which Pax3 can effect downstream molecular and cellular functions in both migrating CNC and cells that ultimately colonize the OFT. We propose that Pax3-mediated specification of CNC must occur very early prior to delamination and emigration from the neural tube. To test this hypothesis, I am using both the Wnt1-Cre (expressed in neural tube/crest) and P0-Cre (only expressed in migratory crest) mice crossed into the ROSA26-lacZ reporter (R26lacZ) background to lineage-trace CNC in a Pax3-deficient background. This dual marking system allows us to mark E8-8.5 CNC while still in the neural tube (before delamination) and compare this with CNC that are marked only after they have exited the neural tube. Additionally, to verify the relative contribution of Wnt1-Cre vs. P0-Cre expressing CNC cells, we crossed both Cre lines to the ROSA26-eGFP-DTA (R26DTA) mice, which will enable us to spatiotemporally ablate CNC at different times during development. This enables us to definitively assess the specific spatiotemporal requirement of CNC during in utero cardiogenesis.

108. Global physiologic and genomic assessment of catecholamine-deficient embryonic mouse hearts suggests novel interactions with calcium, NF-1, and retinoic acid signaling pathways
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The catecholamine hormones norepinephrine (NE) and epinephrine (EPI) are essential for cardiovascular development; however, little is known about the specific role(s) these hormones play. To evaluate how catecholamines critically influence cardiovascular development, we adopted a “global” physiologic and genomic approach using mice in which the gene responsible for NE and EPI production, dopamine beta-hydroxylase (Dbh), was disrupted. High resolution ultrasound imaging (Visualsonics Vevo 660 with 40 and 55 MHz transducers) was used to measure physiologic heart rates from Dbh-/- and Dbh+/+ embryos in vivo. Dbh-/- embryos survive through E10.5 with little or no detectable abnormalities. However, roughly 50% of the Dbh-/- embryos die abruptly between E10.5 and E11.5. Surprisingly, of all embryos alive through E11.5, there is no significant difference in heart rates for Dbh-/- mice compared to controls in vivo. Thus, aside from the abrupt mortality between E10.5 and E11.5, there is no other physiologic indication that catecholamine-deficient embryos are in jeopardy. We therefore explored a genomic strategy whereby RNA was isolated from E10.5 Dbh-/- and Dbh+/+ hearts (n=4 each) that were confirmed via ultrasound imaging to be viable and beating at similar rates in vivo. The RNA was purified, amplified, and analyzed for gene expression differences using Affymetrix Mouse Genome 430A 2.0 Arrays. Of the >22,000 genes on this array, only 23 showed a significant ($P<0.05$) change that was >2-fold in magnitude. Preliminary analysis of these data suggest that genes associated with Ca2+,

neurofibromatosis-1 (NF-1), and retinoic acid signaling pathways are likely influenced by catecholamines in the embryonic mouse heart. We are currently investigating the mechanisms underlying these novel and potentially important interactions.

109. Discovery and functional analysis of novel genes required for zebrafish heart development through microarray techniques

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Approximately 1% of newborns are born with structural malformations of the heart. However, the genetic pathways leading to CHD are still poorly understood. In order to study the genes which are essential for normal morphogenesis of heart, especially late steps such as endocardial cushion and valve development, we have utilized gene chip microarray techniques to identify novel genes. Using the wild-type AB strain carrying tie2::EGFP and flk1::EGFP transgenes, we dissected whole hearts from the remainder of the body, created a single cell suspension, and separated GFP+ from GFP– cell populations. Total RNA was then extracted and Affymetrix gene chip microarray was performed. Initial microarray data identified 792 transcripts which were expressed only in endocardium and 1385 transcripts expressed only in the myocardium of tie2::EGFP zebrafish hearts. In flk1::EGFP zebrafish, 428 genes were detected only in endocardium, and 282 genes only in the body blood vessel endothelium. Combining analysis of the two above microarray data sets, 52 genes were found to be expressed in the endocardium exclusive of the myocardium or any other part of the body (including endothelium). We chose 12 interesting genes (IG) for further analysis. RT-PCR confirmed that all were present in GFP+ but not GFP- cells. Whole-mount in situ hybridization with the IGs also shows specificity of signal in the heart region. Gene knock-down by morpholino injection shows that estrogen receptor 1 (ER1) has a negative regulatory role in cushion formation and valve elongation and possibly functions to limit EMT to the AV boundary while suppressing EMT in the atrial and ventricular chambers. In addition, our analysis suggests a role for Aquaporin-8 in blood vessel organization and development. Thus, our technique appears to be a powerful method for identifying novel genes and pathways affecting late heart development, and which may contribute to human pathology.

110. Electrophysiological Characterization of the Chicken Heart Outflow Tract

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Formation of the heart involves a precise series of molecular and morphogenetic events during the development of an embryo. Heart morphogenesis occur in two consecutive steps, initially the chambers are formed followed by septation. Cardiac septation involves the closure of direct communications between left and right atria, ventricles and subarterial channels, and the development of the right atrioventricular junction and left ventriculoarterial junction. Septation is basically remodeling of the heart from a single-channel peristaltic pump to a dual-channel, it takes place in the embryos that are 3 days old (St-20) in incubation. In general heart morphology in early stages of development is pretty much understood. However the events and mechanism involved in heart formation remains to be elucidated. In particular there is no information about the electrical activity of the outflow tract. Thus we perform intracellular recordings (Action potentials) of spontaneous heart beating from the outflow tract (also known as bulbus cordis) from stages 18-32 and study the pattern of APs and relate those to the morphological changes previously described. We have identified three different types of AP's in the different regions of the outflow tract: bell, triangular and elongated ventricular-like shaped. The sepatation of outflow tract initiates around stages 21-24 with endocardial cushions forming on the either side of outflow tract proximal right (PR), proximal left (PL) and at the distal right ventral (DRV), the proximal left (PL) having an adjacent area with electrical activity which is very similar to ventricular (V) or atrioventricular (AV) canal, but with a longer duration than the latter (elongated ventricular-like shape). Associated with these morphological changes we have identified that the APs of the area close to the distal right ventral cushion (DRV) exhibited a bell shape. In stages 25-28 a new endocardial cushion

had developed in the distal segment of the bulbus, distal left ventral (DLV) this display an adjacent region with action potential that has triangular shape. At this stage the proximal left is an extension of superior atrioventricular cushion that can be established from duration of action potential (AP) that increases as we travel from proximal to distal along this cushion. In stages to follow the intercalated endocardial cushions were becoming apparent, each of the cushions were developing into aortic and pulmonary halves of the distal segment. Thus we have characterized the electrical activity in the outflow tract in various stages of development and correlate the morphological changes with specific electrical activity. Our future objective is to incorporate ion channels composition and their properties that are accountable for the development of the electrical activity in the different regions of out flow tract. Supported by LPP-AHA-SDG 0530140N.

111. Alterations in Wnt5a Affect Cardiac, Skeletal and Craniofacial Development

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The Wnt family of genes is a large group of cell-cell signaling molecules that are important in human development and disease. We have recently shown that mice homozygous for null mutations in Wnt5a have a 100% incidence of cardiac malformations, most commonly persistent truncus arteriosus. These mice also have severe craniofacial and limb abnormalities, which are similar in form to those seen in human Robinow syndrome, a hereditary craniofacial/skeletal/genital and cardiac syndrome. The more phenotypically severe autosomal recessive Robinow syndrome is associated with mutations in the orphan tyrosine kinase receptor ROR2, however no cause has been discovered for the autosomal dominant form of Robinow syndrome. Here, we show that a heterozygous mutation in the coding region of Wnt5a resulting in an amino acid substitution at a highly conserved cysteine residue in exon 4 is associated with autosomal dominant Robinow syndrome in all 7 affected members of the family initially described by Dr. Robinow in 1969. The single living unaffected family member has two wild type alleles. Linkage analysis supports a role for Wnt5a in the causation of this disorder and excludes other loci containing genes associated with the Wnt5a signaling pathway. A second unrelated patient with dominant Robinow syndrome has been found to have a different Wnt5a mutation in exon 3, resulting in an amino acid substitution at another conserved cysteine residue. Functional analysis of these sequence variants of Wnt5a has shown a reduction in Wnt5a activity using a zebrafish wnt5-dependent cell migration assay. This data suggests that a Wnt5a signaling pathway dependent on ROR2 for signal transduction is important in human craniofacial, skeletal and potentially cardiac development, and that proper formation of these structures is sensitive to variations in Wnt5a function.

112. A Forward Genetic Screen for Mouse Mutants with Cardiovascular Morphogenesis Defects

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Congenital heart disease (CHD) is the most common birth defect and a significant cause of morbidity and mortality in humans. Molecular identification of genetic mutations causing human CHD is hampered by pre-reproductive lethality, incomplete penetrance, variable expressivity. In an effort to identify new CHD candidate genes, we have performed a forward genetic screen in mice for mutants with cardiac morphogenesis defects analogous to human CHD. We hypothesized that the fetal to perinatal circulatory transition selects for the structural heart defects observed in human CHD, and therefore we designed a screen to ascertain heart defects in mice that fail the perinatal circulatory transition. Our screen utilized ENU as a mutagen and a three-generation backcross to identify and maintain recessive mutations. We used a hierarchical screen to identify perinatal lethals and then cardiac anatomic evaluation to ascertain structural heart defects within lethals. ENU mutagenesis resulted in a 5-fold induction of perinatal lethality, with 7.5% of live-borns from the mutagenized population vs. 1.5% from control non-

mutagenized dying within 5 days of birth. Structural heart abnormalities have been observed from 40 of 214 independent lines and in greater than 15% of perinatal lethals analyzed to date. Specific defects include atrial septal defects, ventricular septal defects, common atrioventricular canal, double-outlet right ventricle, truncus arteriosus, and interrupted aortic arch. All of the identified structural defects have a direct analogue among human CHD defects. Mutations in five lines, each resulting in atrioventricular septal defects, have been mapped. The cac1 mutation, causing complete common atrioventricular canal, preaxial polydactyly and cleft palate, has been positionally cloned. The cac1 mutation causes abnormal splicing of IFT172, a cilia biogenesis gene. This work identifies a previously unknown role for cilia machinery in cardiac development.

113. The Role of the Extracellular Matrix in the Development of the Electrical Activity of the Heart

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The heart is the first organ to form during embryogenesis, and its function is critical for the proper development and survival of the embryo. It is well established that heart precursors are generated within bilateral fields in the lateral mesoderm and then converge toward the midline to form a beating linear heart tube. In the chicken the process of fusion of right and left primordia occurs at 32 to 35 hrs of incubation, and 2 hrs later the tubular heart is formed. At this time, the heart is composed of a uniform epithelial layer, two myocardiac cells thick, which lies on the abundant extracellular matrix (ECM) known as cardiac jelly, which separates this epithelium from the endocardium. During cardiac development the distinct heart regions display differences in the action potential configuration, which are correlated with distinct molecular phenotypes. It has been well documented that important electrophysiological changes occur during the embryonic development of mammalian and avian hearts. In recent years some studies have demonstrated that ion channel expression, as well as ion transporters, can be age- and chamber-specific in the developing mouse heart. However, little is known about the role of the ECM factors in the differentiation of the electrical activity of the different regions of the developing heart. We hypothesized that components of the extracellular matrix (ECM) in the cardiac cushions (CC) are able to modulate the shape of the cardiac action potential (AP). To test our hypothesis we perform intracellular recordings of spontaneous action potentials (AP) from intact atrium (A), atrio-ventricular canal (AVC) and ventricular (V) areas from 4 day old embryonic. Each area of the heart A, AVC and V exhibits distinct electrical signatures. Similar AP phenotypes were observed in organotypic cultures of A, AVC and V tissues from 3 day (E-3) old embryonic heart + 24 hrs in culture. However, co-culture of CC isolated from E-3 chick cardiac embryo for 24 hrs, in direct contact with atrial or ventricular tissue from the same heart, caused a modification in the AP characteristics of these tissues. These changes were characterized by an increase in the duration of the APs and a decrease in the maximal rate of rise. These changes were more profound in cells localized closer to the cardiac cushion tissue and diminished gradually as the distance from the cardiac cushion increased. Furthermore our preliminary studies show that two important components of the cardiac ECM, fibronectin and laminin can influence cardiac electrical behavior. These observations suggest that components of the ECM have the potential to modify or modulate ionic channel and/or transporters expression, in cardiac cells, and thus may play a role in determining cardiac cell phenotype. The objective of the proposed study is to further document these observations, and to determine the specific channels and/or transporters that are induced or modified in cardiac myocytes upon contact with the cardiac cushions during development, and how the regional electrical specification is determined during early heart formation. Supported by LPP-AHA-SDG 0530140N

114. Cardiovascular Defects in a Murine Model of Neural Crest Ablation

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Neural crest cells are thought to play a critical role in the morphogenesis of the human conotruncus, as well as in the morphogenetic processes that result in conotruncal heart defects. Seminal studies in avian embryos have shown that ablation of the neural crest results in cardiac outflow tract defects and aortic arch patterning abnormalities. However, avian and mammalian cardiovascular patterning differ in several important respects. While fate mapping experiments in mice suggest a conservation of function, the functional requirement for these cells in cardiovascular development in mammals has not been formally tested. We used the PuΔTK selector mouse line, which expresses a truncated version of the herpes simplex virus-1 thymidine kinase (TK) after Cre-recombination. Ganciclovir (GCV) inhibits DNA synthesis in cells that express TK by competitively inhibiting the incorporation of guanosine into the elongating DNA, resulting in cell death. We crossed this line with the Wnt-1 Cre line, which expresses Cre-recombinase in pre-migratory neural crest cells. By administering GCV intraperitoneally to pregnant dams between 7.5 and 9.5 days post coitum we were able to ablate neural crest cells in PuΔTK:Wnt1-Cre embryos. Affected embryos displayed a spectrum of cardiovascular defects including truncus arteriosus, double outlet right ventricle, varying degrees of dextroposition of the aorta, and ventricular septal defects, as well as aortic arch and coronary artery patterning abnormalities. We show that the severity of the cardiovascular phenotype is directly related to the level and extent of neural crest ablation. This is the first report of neural crest ablation in mammals, and provides important insight into the role of the mammalian neural crest during cardiovascular development. These findings also provide additional support for the theory that abnormalities in neural crest cell migration or function play a critical role in the pathogenesis of conotruncal heart defects in humans.

115. The effects of fluid forces in regulating cardiac valve development

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In previous studies our group has described a morphological model of cardiac valve formation. In this model, atrioventricular (AV) valve leaflets form within a 3-D tubular scaffold. AV leaflet formation occurred only when co-cultured with cardiac myocytes. Tube scaffolds seeded with cardiac myocytes spontaneously contract as a syncitum, and thus generate a low level of fluid flow. This led us to test the hypothesis that fluid flow regulates valve leaflet formation. To test this hypothesis we created a fluid flow bioreactor in which defined levels of flow could be generated. When cultured under low flow conditions (Reynolds Number, Nre = 0.1), leaflet structures were observed within the lumens tubes that contain AV explants and without cardiac myocytes. When cultured under higher flow conditions (Nre=5) no leaflets were observed despite the presence of cardiac myocytes. When flow is restricted, AV explants amorphously expand into web like cell aggregates. The regulation of extracellular matrix molecules was also explored in this model. Here the role of periostin was explored using sense and antisense expressing adenoviruses. Explants infected with the sense periostin virus showed expanded acellular regions and large depositions of ECM proteins including type I collagen and tenascin. In contrast, those explants that were infected with the antisense periostin virus had relatively compact leaflets that, interestingly, contained cells that expressed muscle markers (MF-20). Finally, atomic force microscopy (AFM) was used to determine the inherent stiffness of cardiac valves. Stiffness was determined for both the AV leaflets and chordae tendineae for wild type and periostin null mice. There was a decrease in stiffness in chordae tendineae of periostin null mice. Leaflets of the perisotin null mouse showed a reduction in the stiffness of the valve (>50%). These changes correlated to a change in the surface morphology observed with the AFM using the scanning mode.

116. Expression of Extra-cellular Matrix Proteoglycans in Developing Heart

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Background: Extra-cellular matrix (ECM) proteoglycans have always been implicated in the developmental processes. While some of them (aggrecan, versican) have long been found to reside in the heart, there roles in early stages of atrio-ventricular valve (AV valve) development has hardly been studied. We have studied the expression pattern of five choindroitin sulfate (CS)/ Dermatin Sulfate (DS) containing proteoglycans in the heart of 2 day old zebrafish. Proteoglycan expression in early stages of heart development: We have studied the expression pattern of five CS/DS containing proteoglycans in 2 d old zebrafish heart. The proteoglycans studied are the two hyalectans (aggrecan and versican), two small leucine-rich proteoglycans (decorin and biglycan) and syndecan-2. Immuno-histochemistry with versican antibody shows that versican is present in the AV region of the developing heart. We have also performed whole mount and section in-situ using probes specific for zebrafish for these five proteoglycan mRNA. Whole mount- in situ shows a generalized pattern of expression of these proteoglycans in the heart. Section in-situ shows that decorin is strongly expressed in the mesenchymal layer all along the heart while versican is expressed in AV boundary region of the developing heart. Inference: The expression of CS/DS proteoglycans in the developing stages indicates that they might play a role in the development of AV valve and the heart. Further experiments will better elucidate their roles in AV valve and heart development. Future work: Future work involves using anti-sense morpholino against proteoglycans to knockdown their expression and study the phenotype in heart. We will also look at the interaction between the proteoglycans and the signaling pathways implicated in the process of development of AV valve.

117. Differentially expressed genes related with Galpha13 pathway in the cardiac outflow tract tissue of Cx43 knockout mice

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Objective:To investigate the genes involving in the pathogenesis of pulmonary outflow tract (OFT) obstruction in Cx43 knockout mice. Methods: The cDNA was retrotranscripted from RNA extracted from OFT tissues of both Cx43 knockout and Cx43 wildtype mouse embryos on ED14.5. The biotin-labeled cRNA derived from the transcription of cDNA was fragmented as probes. The probes were hybridized with Affymetrix Mouse Genome 430 2.0 Array. Gene Array Scanner was used to screen the signals of hybridization and the expression of genes was detected. Some of the differentially expressed genes were identified using real time quantitative RT-PCR. The expression level of Rock-1 and α-SCA proteins were detected by immunohistochemical analysis from ED11.5 to ED15.5. Results: There were 288 genes upregulated and 199 downregulated in Cx43 knockout OFT tissues compared with Cx43 wildtype. Functions of proteins encoded by the altered genes encompassed all functional categories, with largest percentage in genes involved in regulation of transcription and cell cycle, etc. Interestingly, Rock-1, a gene at the downstream of Galpha13 pathway, was observed much less expressed in the Cx43KO mice OFT tissues on both mRNA and protein levels. This was highly consistently with the delayed process of myocardialization of the proximal OFT septum in the Cx43KO mice, which was observed most obviously during ED13.5 to ED15.5. Conclusions: Our studies indicate that genes related with Galpha13 signaling pathway may be involved in the pathogenesis of pulmonary outflow tract obstruction in Cx43 KO mice. Downregulation of Rock-1 may play an important role in the delayed process of myocardialization in the Cx43KO mice.

118. A Rare Human Sequence Variant Reveals MYOCARDIN Autoinhibition

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Congenital Cardiovascular Malformations (CCVM) occur in approximately 1% of all live births and are the leading cause of mortality in the first year of life, but the genetic etiologies for most CCVM remain unknown. Utilizing a candidate-based screen of humans with CCVM to search for genetic mutations of developmentally important cardiac genes, we found a rare sequence variation in the cardiac and smooth muscle co-activator, Myocardin (MYOCD). This sequence variation converted an evolutionarily conserved lysine in the basic domain of MYOCD into an arginine (K259R). Molecular analysis of this mutation in the cardiac isoform of MYOCD revealed that it produced a hypomorphic protein that had reduced affinity for its cofactor, serum response factor (SRF). Curiously, the smooth muscle isoform of MYOCD that lacks an amino-terminal domain (NTD) was functionally normal even in the presence of the K259R mutation. We found that the NTD functions by binding to the SRF and MEF2 interacting domains within MYOCD resulting in attenuated MYOCD-dependent transcription both in vitro and in vivo. Further analysis of the NTD's activity revealed slightly stronger inhibition of MYOCD K259R, which may explain the hypomorphic function. This work reveals a novel mechanism by which MYOCD may regulate the SRF and MEF2 transcriptional programs.

119. The N-terminus of FOG-2 Interacts with the MTA Proteins of the NuRD Complex to Mediate Transcriptional Repression

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FOG-2 is a transcriptional co-repressor that is required for proper cardiac morphogenesis as mice deficient in this factor die during mid-gestation of cardiac malformations. FOG-2 interacts with GATA4 and through this interaction attenuates GATA4's ability to activate cardiac specific gene expression. The first 12 amino acids of FOG-2 (the FOG Repression Motif) are necessary to mediate this repression. To determine the mechanism by which the FOG Repression Motif represses transcription, we took a biochemical approach. Using MALDI-TOF mass spectrometry, we identified 7 polypeptides from rat neonatal cardiac nuclear extracts that co-purified with a FOG-2-GST fusion protein. All proteins identified are members of a nucleosome remodeling complex called the NuRD complex. To determine which of the NuRD subunits directly interacts with the FOG Repression Motif, in vitro binding assays were performed. These assays demonstrated that Metastasis-Associated protein (MTA)-1, 2 and 3 interact directly with the first 12 amino acids of FOG-2, but not with a mutant form of FOG-2 that is unable to repress transcription. Co-immunoprecipitation using an anti-FOG-2 antibody confirmed FOG-2 and MTA proteins interact in vivo. Further in vitro binding assays revealed that a novel domain located near the C-terminus in all MTA proteins mediates their interaction with FOG-2. Using transient transfection assays, we show that knockdown of MTA protein levels in NIH 3T3 fibroblasts results in a dramatic reduction in the ability of FOG-2 to repress transactivation of a GATA4-dependent promoter. Finally, in situ hybridizations demonstrate that MTA1, MTA2, and Mi2β are expressed in the heart during development and appear to be enriched within certain regions of the heart. Together, these results suggest that FOG-2 recruits the NuRD complex to GATA4-activated promoters to repress transcription during heart development.

120. The wayward journey of the PE cell: A comprehensive, multi-dimensional, study of proepicardium migration

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The proepicardium (PE) is a unique structure which arises from mesothelium of the septum transversum. The PE translocates to the heart where it forms the epicardium and progenitor cells of the coronary vessels. The epicardium covers and protects the myocardium, while the coronary vessels provide blood flow throughout the entire heart. Despite these vital roles for PE descendants, the process in which PE cells translocate to the myocardium in mammals is not well defined. The current paradigm states that cellular cysts of PE float across the pericardial space and contact the outer surface of the myocardium at the atrial-ventricular sulcus. However, this definition does not provide a satisfactory explanation for the directionality or localization of PE migration. Our studies are focused on establishing a better understanding of the mechanism for PE migration from the septum transversum to the heart within the mouse model system. We performed a detailed histological and immunohistochemical survey in combination with electron microscopy and ex vivo studies to define the timing, size, and mechanics of PE migration. We provide thorough documentation of PE development that redefines the size of the PE migratory field and the mechanism of migration. We describe PE migration as a combination of differential growth and expansion. PE cells migrate towards the heart predominantly through the use of multicellular villi. Due to PE expansion, heart growth, and motion created by the beating heart, PE cells detach from villi once in contact with the myocardium by a "Velcro type" mechanism. The presence of floating cysts is limited to the areas of greatest distance between the PE and the developing heart. Thus, our observations are the first to define direct contact between the PE and the myocardium as an element of proepicardium migration.

121. Myofibrillogenesis in zebrafish

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To determine whether the process of myofibril assembly in a live animal corresponds to that seen in tissue culture, we have begun to use the zebrafish model system where the temporal progression of myofibril formation can be followed in a single fish in myotomes that form sequentially along the flank. Antibodies to sarcomeric alpha-actinin and muscle myosin II were used to stain embryos that were fixed at 20 - 25 hours post-fertilization. Small periodic bodies of alpha-actinin (z-bodies) were arrayed along actin fibers in the youngest myotomes in a pattern that appeared identical to premyofibrils first discovered in avian cardiomyocytes. In young myotomes, muscle myosin was present in overlapping bundles that resembled the myosin patterns in avian nascent myofibrils. A-bands and Z-bands were present in mature myofibrils in the elongated cells in older myotomes. However, at the ends of these cells the patterns of alpha-actinin and muscle myosin resembled the arrangement seen in younger myotomes, suggesting that as the cells elongated, premyofibrils were involved in the extension of myofibrils that accompanied cell growth. For live cell observations, plasmids with a zebrafish skeletal muscle alpha-actin promoter encoding YFP-sarcomeric alpha-actinin or –FATZ, -myotilin or -skeletal actin were injected into the yolk right after fertilization or into one of the first two blastomeres. Mosaic transgenic zebrafish displayed the expected localization of alpha-actinin, FATZ, myotilin and actin in the young and old myotomes even after several days. Fluorescence Recovery After Photobleaching (FRAP) measurements showed that YFP-alpha-actinin, -FATZ, -actin, and –myotilin measured in the Z-bands had recovery profiles similar to those found for Z-bands in avian muscle cells grown in tissue culture. The FRAP properties of these Z-band proteins reflect the dynamic nature of sarcomeric proteins needed for the formation and maintenance of the myofibril in cardiac and skeletal muscle cells.

122. Human Embryonic Stem Cell Derived Cardiac Myocyte Function: Electrical and Calcium Handling Properties

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There is scant information regarding the development of excitability and Ca handling in early stage human cardiac tissue due to obvious ethical and practical reasons. The establishment of a cardiomyocyte (CM) differentiating system from human embryonic stem cells (hES) may provide a unique model enabling the study of this developmental process. In this study we evaluated the hypothesis that the earliest stages of hES-CM contractile function are accompanied by excitability and Ca handling properties with molecular components that are utilized by mature working myocytes. We tested the hypothesis that hES-CM Ca handling at the earliest stages of beating (12-40 days post-differentiation) is fundamentally the same as that in mature CMs lacking transverse-tubules. A combination of approaches including RT-PCR, immunocytochemistry, whole-cell voltage-clamp, and simultaneous patch-clamp/laser scanning confocal Ca-imaging and surface membrane labeling with Di-8-ANEPPS were employed. We determined that sarcoplasmic reticulum (SR) Ca release channels, RyR2, are expressed in hES-CMs differentiated <40 days in vitro. Store Ca function was manifested as periphery-initiated action potential (AP)-induced Ca transients that spread throughout the cell by propagation of internal Ca release. Cells exhibited Ca sparks that were associated with surface membrane. Caffeine-releasable store Ca was demonstrated, as early as 12 days post-differentiation, with the initiation of spontaneous beating, by focal, temporally-limited puffs of caffeine. Finally, ryanodine decreased the amplitude of spontaneous Ca transients. In summary, hES-CMs possess functional SR Ca stores corresponding with intracellular Ca transients, similarly to mature CMs that lack well-ordered t-tubules. These data establish the presence of functional SR in immature hES-CMs, and together with our previous electrophysiological and contractile protein characterizations suggests that hES-CMs recapitulate functional differentiation expected for working CMs.

123. Phosphorylation of p38 MAP kinase in the embryonic heart under increased hemodynamic stress: a possible role for myocardial growth during development

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Hemodynamic stress is a major epigenetic regulator of heart development. Experimentally increased hemodynamic stress by conotruncal constriction (CTC) during early development results in condensed myocardial layers which indicate accelerated cardiomyocyte proliferation and differentiation. However, molecular pathways responsible for the myocardial changes in CTC-treated hearts remain unknown. We determined increased nuclear accumulation of proliferating cell nuclear antigen (PCNA) 48 hours after CTC treatment (HH Stage 27) in the LV-side of the myocardium, indicating cell cycle regulation is altered by CTC. We next investigated mRNA profiles of the CTC-treated hearts at the same time point using Affymetrix GeneChip. Surprisingly, only <50 genes were either up- or down-regulated with >1.5 fold change, indicating that robust transcriptional regulations do not take place at this time point. We therefore switched focus to post-translational protein modification as a putative mechanism. In order to stably analyze protein modifications in the embryonic heart, surgical protocols of the CTC treatment have been modified. We hypothesized that MAP kinase pathway is regulated by the increased hemodynamic stress because MAP kinase a major mechanotransducer (i.e. hemodynamic responsive). With the modified protocol we tested three major MAP kinases (ERK, p38, and JNK) after 10, 30 and 60 minutes of CTC treatment using immunoblotting. We have determined that p38 MAP kinase was selectively and robustly phosphorylated (>90% increase from baseline, p=0.02, n=7) by the increased hemodynamics stress at 10 minutes and the signals decayed over time. ERK and JNK phosphorylation was not significantly altered (n=7). To our knowledge, these are the first data demonstrating that differential

MAP kinase signaling takes place in the embryonic heart by the increased hemodynamic stress. Of note, p38 MAPK is an upstream regulator of MEF2C, a critical cardiac transcription factor. The p38 MAP kinase may be a key player that regulates hemodynamics-driven myocardial growth, and our protocols will be useful to dissect this pathway in depth.

124. Vessel and blood specification override cardiac potential in anterior mesoderm

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Organ progenitors arise within organ fields, embryonic territories that are larger than the regions required for organ formation. Little is known about the regulatory pathways that define organ field boundaries and thereby limit organ size. Here, we identify a mechanism for restricting heart size through confinement of the developmental potential of the heart field. Via fate mapping in zebrafish, we locate cardiac progenitors within hand2-expressing mesoderm and demonstrate that hand2 potentiates cardiac differentiation within this region. Beyond the rostral boundary of hand2 expression, we find progenitors of vessel and blood lineages. In embryos deficient in vessel and blood specification, rostral mesoderm undergoes a fate transformation and generates ectopic cardiomyocytes. Therefore, induction of vessel and blood specification represses cardiac specification and delimits the capacity of the heart field. This regulatory relationship between cardiovascular pathways suggests new strategies for directing progenitor cell differentiation to facilitate cardiac regeneration.

125. Thymosin b4 is Essential for Coronary Vessel Development and Promotes Neovascularisation via Adult Epicardium

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Coronary artery occlusion is the underlying cause of myocardial infarction (MI). Neovascularisation, an integral component of the cardiac remodelling process to restore blood flow after MI, is limited and insufficient to preserve viable myocardium. Therapeutic approaches for treatment of MI have focussed on replacement of damaged myocardium with embryonic or adult cardiac progenitors and the induction of new vessel formation. Thymosin b4 (Tb4), a peptide implicated in cell migration, minimises cardiomyocyte loss and improves cardiac function after MI. By selectively knocking down Tb4 in the developing heart, we uncovered its essential role in coronary vasculogenesis, angiogenesis and arteriogenesis. We demonstrated that Tb4, secreted from the myocardium, provides a paracrine stimulus to the cells of the epicardium to promote their migration into the myocardium and differentiation into endothelial and smooth muscle cells to form the coronary vasculature. Translation of a vascular development role for Tb4 to that of angiogenic therapy for coronary artery disease in the adult heart relies on the release of the adult epicardium from a quiescent state and restoration of pluripotency. Tb4 treatment of adult cardiac explants stimulated extensive outgrowth of epicardial cells which, as they migrated away from the explant, differentiated into fibroblasts, smooth muscle and endothelial cells, confirming an equivalent vasculogenic potential within the adult epicardium. Adult epicardial cells under the control of Tb4 represent a viable source of vascular progenitors which may be mobilised following cardiac injury for neovascularisation. The role of Tb4 in coronary vasculogenesis may underlie its reported cardioprotection and further elaboration of the mode of Tb4 action will contribute towards the evolution of more efficient therapies for ischaemic heart disease.

126. The Dorsal Mesenchymal Protrusion (DMP) is an Extra-Cardiac Derived Component that Contributes to the Atrioventricular Mesenchymal Complex

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Proper formation and development of the mesenchymal tissues in the developing heart, including the endocardial cushions in the outflow tract (OFT) and atrioventricular junction (AV), and the mesenchymal

cap of the primary atrial septum (CAP), is crucial for normal valvuloseptal morphogenesis. In addition to these, largely endocardial-derived, cell populations, the cardiac mesenchyme also receives contributions from extra-cardiac sources, including the proepicardium and the neural crest. Furthermore, cells associated with the dorsal mesocardium have been shown to contribute to the dorsal mesenchymal protrusion (DMP), located at the venous pole of the heart. The limited availability of specific markers that discriminate the DMP from other AV mesenchymal tissues has made delineation of the DMP difficult and controversial. As a result, the significance of the DMP to AV septation has remained poorly understood. A recent study, however, using the Tie2-Cre/ROSA26RlacZ mouse system, identified the DMP as a non-endocardial derived mesenchymal population. Using this system, and building on the above observations, we performed a comprehensive spatiotemporal study of the location of the DMP with respect to the other AV mesenchymal populations. We show that, during development, the DMP, in combination with the CAP, fuse with the major AV cushions to close the primary atrial foramen and to form the AV mesenchymal complex. In this complex, the DMP constitutes a prominent mesenchymal component, wedged in-between the major AV cushions. This new model for AV septation could provide novel insights into the etiology of congenital cardiac malformations in the venous pole of the heart.

127. Characterization of Crp1Cre mice using both the ROSA26R reporter line and ROSA-EGFP-DTA transgenic mice

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Cysteine-rich protein 1 (Crp1) is thought to regulate actin filament bundling and is expressed in all muscle cell types during embryogenesis and predominately in vascular and visceral smooth muscle cells in the adult. Previously, it was shown that a 5.0kb Crp1 enhancer was sufficient to drive expression in arterial but not venous or visceral muscle lineages. Due to our interests in both outflow tract formation and aortic arch artery remodeling, we used this enhancer to generate Crp1Cre mice for use in lineage mapping, Cre/loxP conditional mutagenesis, and cell ablation studies. By crossing Crp1Cre mice to ROSA26R reporter mice that permanently mark Cre recombinase-expressing cells with lacZ, we can lineage trace the Crp1 expressing cells in utero. Analysis of a developmental series revealed lacZ reporter expression can initially be detected around E10 within the outflow tract of the heart, around the dorsal aorta, and in the aortic arch artery smooth muscle lineages. As development progresses, lacZ expression is expanded, and lacZ was seen in the face, somites, limb muscles, hindbrain, and scattered within the right sided ventricular cells of the embryonic heart. This lineage tracing data is important for future cell ablation and conditional knockout studies to understand where and when Cre-mediated deletion occurs and where Cre-expressing daughter cells finally localize. In order to determine the functional role of the Crp1Cre cells, we crossed the Crp1Cre mice to ROSA-EGFP-DTA mice. Upon Cre-mediated recombination, ROSA-EGFP-DTA mice express diphtheria toxin A (DTA) which results in apoptotic cell death and genetic cell ablation. These mice provide a unique means to assess smooth muscle contribution to aortic arch artery remodeling and outflow tract septation. (Supported, in part, by ICVBM NIHT32 training grant).

128. Complex Structural Heart Defects in Primary Ciliary Dyskinesia

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Primary ciliary dyskinesia (PCD), an autosomal recessive disease characterized by bronchiecstasis and situs abnormalities, is associated with dynein mutations causing ciliary immotility or dyfunction/dyskinesia. Most PCD patients have either situs solitus or situs inversus, with one clinical study showing 6% of PCD patients with heterotaxy syndrome and complex congenital heart defects

(CHD). Through a mouse ENU mutagenesis screen using fetal echocardiography, we recovered a mouse mutation comprised of an in frame deletion of sequences encoding the N-terminus of mouse dynein heavy chain DNHC5. Homozygous mutants exhibit phenotypes consistent with PCD, with some fetuses dying prenatally or neonatally with complex CHDs. To assess the frequency and nature of CHD associated with this dynein mutation, we harvested16 litters of embryos between E16.5 and 18.5. All of the wildtype and heterozygous offspring (89) showed normal body situs. Of the 21 (19%) homozygous mutants obtained, 6 had situs solitus, 7 situs inversus and 8 heterotaxy, with heterotaxy being any situs deviation in the cardiac, pulmonary or visceral anatomy. Further analysis of the heterotaxic embryos showed 3 with levocardia and 5 with dextracardia. Detailed histological analysis using episcopic fluorescence image capture for 2D and 3D reconstructions revealed complex CHD in all 3 embryos with levocardia and in 2 of the embryos with dextracardia. The spectrum of complex CHD included atrial isomerism, superior-inferior ventricles, malposition of the great arteries, AV cushion defects, and azygous continuation of the inferior vena cava. These results indicate a much higher frequency of heterotaxy and complex CHD than previously reported in PCD patients (23% vs. 6%). These findings suggest that complex CHD may be more commonly associated with PCD. Further studies using this animal model may help to elucidate whether ciliary function may have additional roles in situs determination beyond the initial breaking of symmetry at the embryonic node.

129. Role of Pitx2 in myocardial remodelling in the developing and adult heart

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We have generated the myocardial specific Pitx2 knock out mice (komyo), by crossing the Pitx2 floxed mice with the alpha-MyHC Cre mice. Pitx2 komyo mice present cardiac morphological defects, clearly visible from E10.5 during development. The developing komyo hearts present lack of chamber expansion and abnormal remodelling around the inner curvature. As a consequence, fetal and neonatal hearts are grossly misshaped, with enlarged and malformed interventricular septum (IVS) ,abnormal positioning of the left ventricle (LV) outlet, and additionally, absence of the interatrial septum (IAS). Pitx2 komyo animals survive till adulthood but they develop functional impairment, as assessed by echocardiographic analysis. At the cellular level, we have found that the komyo hearts present irregular cellular orientation and organization in the regions which have lost Pitx2 expression. ISH analysis on E12.5 komyo embryos has shown no differences in the expression of cardiac markers and patterning genes within the Pitx2 expression domain and also in the neighbouring (non Pitx2 expressing) cells, thus ruling out a non cell autonomous transcriptional effect of the Pitx2 gene. The only difference was found on BMP10 expression: the normal downregulation of this gene which in wild type mice occurs in the left atrium at fetal stages did not occur in the komyo mice, and left atrial BMP10 expression is retained till the adult stage in the Pitx2 komyo hearts. Our result unravel a crucial role of the Pitx2 gene in cardiac morphogenesis and function.

130. Tbx1 is required for coronary artery patterning

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TBX1, encoding a T-box containing transcription factor, is the major candidate gene for DiGeorge or del22q11.2 syndrome, characterised by craniofacial and cardiovascular defects including tetralogy of Fallot and common arterial trunk. Mice lacking Tbx1 provide a model for DiGeorge syndrome and have severe defects in the development of pharyngeal derivatives including cardiac progenitor cells of the second heart field which contribute to the arterial pole of the heart. The outflow tract of Tbx1 mutant embryos is short and narrow compared to that of wild-type littermates and fails to divide; as a result Tbx1

mutant embryos die at birth with a common arterial trunk. We have carried out a series of genetic crosses using transgene markers of the second heart field and coronary artery endothelial cells to further investigate the Tbx1 mutant outflow tract. Our results suggest that the Tbx1 mutant heart forms in the absence or severe reduction of a specific subpopulation of second heart field cells normally giving rise to subpulmonary myocardium. The Tbx1 mutant ventricular outlet thus has an aorta-like morphology with three valve cusps. In addition, mutant hearts display severe coronary artery patterning defects. During normal development left and right coronary arteries connect with their respective coronary sinuses at the base of the ascending aorta, avoiding subpulmonary myocardium. In Tbx1 mutant hearts two of the three outlet valve cusps appear to be non-coronary and coronary ostia form only at the right/ventral sinus of mutant hearts. As a result, the left coronary artery courses aberrantly across the normally coronary refractory ventral region of the heart. These results suggest that Tbx1 directly or indirectly regulates coronary artery patterning and provide insights into the genetic basis of coronary artery patterning defects.

131. Long form of Latent TGF-beta binding Protein-1 (Ltbp-1L) is essential for cardiac outflow tract septation and remodeling

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Latent TGF-β binding protein-1 (Ltbp-1) is a member of the Ltbp/fibrillin family of extracellular proteins. Due to usage of different promoters, Ltbp-1 exists in two major forms: long (L) and short (S), each expressed in a temporally and spatially unique fashion. Both Ltbp-1 molecules covalently interact with three isoforms of latent Tgf-β and play an important role in latent Tgf-β secretion, extracellular matrix (ECM) deposition and activation. We wanted to explore the in vivo role of Ltbp-1 in mouse development, when only the long isoform is expressed. Hence, we mutated the Ltbp-1L locus by gene targeting. Ltbp-1L null animals die shortly after birth from defects in heart development, consisting of the improper septation of the cardiac outflow tract (OFT) and remodeling of the associated vessels. These cardiac anomalies present as persistent truncus arteriosus (PTA) and interrupted aortic arch (IAA), both of which have previously been associated with faulty function of the cardiac neural crest (CNC) cells. Ltbp-1L is an important component of the ECM in the OFT and pharyngeal apparatus as the CNC cells populate, septate and remodel these embryonic structures into the great arteries and the branching vessels. The lack of Ltbp-1L in the extracellular milieu of the septating heart and associated vessels results in decreased Tgf-β activity and altered gene expression pattern and function of CNC cells. This phenotype reveals a critical role for Ltbp-1L in the regulation of Tgf-β presentation in heart organogenesis.

132. Regulation of Tbx20 by cardiac transcription factors

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The T-box family member Tbx20 plays a fundamental role in patterning the developing vertebrate heart; however the mechanisms of Tbx20 transcriptional regulation are largely unknown to date. We found that TBX20 is significantly upregulated in cardiac tissue of patients with Tetralogy of Fallot (TOF) and report an analysis of underlying regulatory pathways. Investigations of human TBX20 transcripts lead to the identification of two new exons 3' to the known TBX20 message resembling the mouse variant Tbx20a, as well as an extended 5' UTR. Comparative genomics revealed that regions within the first intron, at the transcriptional start site and –2.5 kb upstream are evolutionary conserved and contain several putative binding sites for cardiogenic transcription factors. Within the core promoter region we recognized functional binding sites for TFAP2 transcription factors and identified proteins of the TFAP2 family as repressive regulators of the TBX20 gene in-vitro and in-vivo. Interestingly, we found that TFAP2C is significantly downregulated in tissue samples of patients with TOF, providing a possible explanation for

the overexpression of TBX20. Mutation analysis did not reveal any disease causing sequence variations in TBX20 and the DNA binding domain of TFAP2C, suggesting that mutations in these proteins are unlikely to be a major cause of TOF. Employing Chromatin-Immunoprecipitation (ChIP) and siRNA knockdown in the HL-1 mouse cardiac cell line we furthermore identified GATA4, Mef2, Nkx2-5 and SRF as direct regulators. These findings point to a positive feedback loop within the core cardiac transcription factor network, as Tbx20 is known to physically interact with Nkx2-5 and GATA4 and to activate cardiac enhancers of Nkx2-5 and Mef2c in-vitro. To summarize, we provide for the first time insights into the regulation of Tbx20 by transcription factors involved in cardiac development.

133. The PDZ Binding Motif and Interaction of GIPC with the Type III TGFβ Receptor (TGFBR3) are Required for Noncanonical TGFβ Signaling

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Transforming Growth Factor β (TGFβ) plays key roles in cardiovascular development and homeostasis. Two classes of receptors bind TGFβ. The first class consists of two serine/threonine kinase receptors termed Type I (TGFBR1) and Type II (TGFBR2). The second class contains two transmembrane proteins termed the Type III TGFβ receptor (TGFBR3), or betaglycan, and endoglin. TGFBR3 binds all TGFβ ligands with similar affinity and can present ligand to the TGFBR2/TGFBR1 complex. Gain- and loss-of-function experiments have demonstrated a requirement for TGFBR3 in TGFβ-stimulated epithelial mesenchymal transformation (EMT), a necessary step in heart valve formation. Targeted deletion of Tgfbr3 in the mouse results in a spectrum of cardiovascular defects, most strikingly, a failure of coronary vessel development. TGFBR3 has a short 43 amino acid cytoplasmic domain that is not required for ligand presentation. Here we demonstrate an absolute requirement for the cytoplasmic domain of TGFBR3 for EMT in vitro. Overexpression of wildtype TGFBR3 in normally nontransforming ventricular endocardial cells, followed by ligand addition, results in gain-of-function (EMT). Overexpression of TGFBR3 lacking the entire cytoplasmic domain lacks this activity. The three C-terminal amino acids – STA - are required for binding to the PDZ domain containing protein, GIPC. GIPC has been shown to stabilize TGFBR3 on the plasma membrane. Overexpression of a TGFBR3 mutant lacking these three amino acids abolished TGFBR3-mediated EMT in ventricular endothelial cells demonstrating an absolute requirement for these amino acids. Overexpression of GIPC in atrioventricular (AV) cushions enhanced EMT (175% of control), consistent with a requirement for GIPC interaction with TGFBR3 to mediate EMT. Recent targeting of synectin (GIPC) in zebrafish and mouse have revealed a role for GIPC in arterial patterning. Together, these data demonstrate that the PDZ binding motif of TGFBR3 and GIPC interaction with TGFBR3 function to regulate endothelial cell behavior. Supported by HL52922, HL67105, & AHA06551.

134. Redundant roles of Inositol 1,4,5-trisphosphate receptors for formation of endocardial cushion and myocardial wall

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Intracellular calcium signaling plays an essential role in cardiomyocyte physiology and gene expression, however its function in cardiac development remains largely unknown. Inositol 1,4,5-trisphosphate receptor (IP3R) is an intracellular calcium release channel located on the endo/sarcoplasmic reticulum and one of components in intracellular calcium signaling molecules. We have found that all three subtypes of IP_3R (IP3R1, 2 and 3) are expressed during cardiogenesis and that IP_3R1 and 2 double knockout (DKO) mouse embryos demonstrate thin myocardial wall and impairment of epithelial-mesenchymal transformation in atrioventricular canal, resulting in embryonic lethality around embryonic day (E) 11. In the endocardium of atrioventricular canal in DKO embryos, translocation of a transcription factor NFATc into the nucleus was inhibited at E9.5, suggesting that IP_3R1 and 2 mediate intracellular

calcium signaling upstream of calcineurin-NFAT signaling, which is essential for valvuloseptal development. In an effort to identify possible molecular defects in DKO mutant myocardial wall, we compared the gene expression profiles of wild-type and mutant hearts at E9.5 by microarray analysis. According to our selection criteria for signal strength, expression ratio, and reproducibility, several cardiac genes, including a gene encoding T-box transcription factor, Tbx5, were upregulated in mutant hearts. In situ hybridization analyses further confirmed upregulation of Tbx5 in DKO mutant myocardium, and demonstrated upregulation of ANF, a known downstream target of Tbx5. In cell proliferation analysis the number of BrdU-positive or phospho-Histone H3-positive cells was dramatically decreased in DKO ventricular myocardium around E9.5 compared with wild type heart, while apoptotic signals detected by TUNEL assay were not significantly altered. These results suggest that IP_3R1 and 2 may play a role in development of the ventricle, through a regulation of Tbx5 expression, that is essential for maintenance of proper cell proliferation and maturation of ventricular myocardial wall.

135. The Role Of Tie1 In Cardiac Valve Ontogeny

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Tie1 is an endothelial specific receptor tyrosine kinase (RTK) required for developing stable vasculature, endothelial cell survival and quiescence. Global knockouts of Tie1 result in embryonic lethality around E13.5 due to vascular defects. To date, no ligand for Tie1 has been identified, and downstream targets of receptor activation remain unknown. It is suggested that Tie1 signaling is dependant on an association with Tie2, a structurally related endothelial RTK, though we hypothesize that Tie1 signals in a context dependent, Tie2 independent pathway. Our laboratory has developed a Cre transgenic line (NFATc-P2Cre) specific for pro-valve endocardium. Using a floxed Tie1 allele, we have deleted Tie1 specifically in this region, allowing us to bypass the requirement for Tie1 in extracardiac vascular development. Our data suggest that loss of Tie1 in the valves leads to defects in valve integrity and remodeling. We will examine alterations in proliferation, apoptosis, and matrix production/ deposition to identify potential factors contributing to the thick valve phenotype in Tie1 conditional knockouts. We are also creating a floxed Tie2 allele using BAC recombineering to conditionally knockout Tie1 and Tie2 in pro-valve endocardium to elucidate the function of potential Tie1/Tie2 interactions in the developing mammalian heart.

136. Identification and Functional Characterization of Cx45α7 Promoter

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The sinoatrial node (SAN) is the primary pacemaker of the heart and drives the cardiac conduction system. In adult mouse, SAN is defined as a heterogeneous population of automatic cells near the Crista-terminalis (CT) with its core positive for Cx45α7 and periphery positive for both Cx45α7 and Cx43α1. While the Cx43α1 promoter has been well characterized and shown to be modulated by known myocardial transcription factors, the Cx45α7 promoter and its regulation is unknown. We sought to identify the promoter and its regulation of Cx45α7 as a means to understand the development of the SAN. Developmentally Cx45α7 is expressed through out the primitive heart but is progressively restricted to the proximal conduction system especially the SAN and the AVN by adulthood. Cx45α7 has multiple isoforms and hence we identified three putative promoters elements (PPE1-3) based on the isoform information and conservation among species. We also found three conserved enhancer elements (CEE1-3) identified using bioinformatic techniques (rVista, Trafac, Cismols) and conservation across species and among the other cardiac connexins. These elements were tested using in-vitro reporter assay in five different cell types. We have identified a promoter (PPE1) that regulates the cardiac isoform of Cx45α1 and a strong enhancer element (CEE1) that might regulate Cx45α1 in multiple organ systems. In contrast to Cx43α1 which is repressed by pro-conduction cardiac transcription factors and activated by pro-

working myocardium transcription factors, the promoter and the enhancer of Cx45α1are positively modulated by pro-conduction cardiac transcription factors and inhibited by pro-working myocardium transcription factors variably in all cell lines tested. In vivo studies of these promoter/ and enhancer elements should provide insights into the developmental regulation of Cx45α7 expression.

137. Sinoatrial node formation from caudal cardiac progenitors requires Tbx18

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The sinoatrial node (SAN), the pacemaker of the heart, is located at the junction between the right atrium and the superior caval vein. The cellular origin and mechanism of formation of the SAN have not yet been clearly defined. The developing SAN consists of a body within the right sinus horn bordering the atrium, and an extension in the venous valves. The entire primordium expresses Tbx3 and Hcn4. The sinus horns express Tbx18 and are formed by Tbx18-dependent recruitment and myocardial differentiation of mesenchymal precursors. Consistently, we found that the SAN body within the sinus horn expresses Tbx18. To investigate the role of recruitment in SAN formation, Tbx18-deficient mice were studied. We found that Tbx18 mutant embryos initially developed only the Tbx3-positive, Tbx18-negative venous valve part of the SAN. The entire SAN body of the sinus horn was lacking. From ED12.5 onwards, the caval veins of mutants myocardialize independently from Tbx18, which allowed the formation of a SAN-like structure that runs a nodal gene program. At ED17.5, Nkx2-5 expression was found in the entire SAN-like structure, whereas in control hearts only the part bordering the atrium expressed Nkx2-5. The pacemaker channel Hcn4 showed a diffuse expression pattern which might be caused by the aberrant presence of Nkx2-5 in mutant SANs. Throughout development the mutant SAN was much smaller. However, the rate of proliferation of the Tbx18-positive cell population was not different, and no apoptosis was observed. These data indicate that the main process of SAN formation is by Tbx18-dependent recruitment of mesenchymal precursors, and that the Tbx18-independent phase of myocard formation can not compensate for the failure to form most of the SAN.

138. Altered hypoxia inducible factor-1 alpha levels correlate with coronary artery anomalies

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Previously we showed that hypoxia, as assessed by the hypoxia indicator EF5, is highest in the outflow tract (OFT) myocardium and other regions corresponding to the location of the major coronary vessels of the developing chicken heart. The EF5 positive tissues were also specifically positive for nuclear-localized hypoxia inducible factor-1 alpha (HIF-1a), the oxygen-sensitive component of the hypoxia inducible factor-1 (HIF-1) heterodimer. In this study we altered ambient oxygen levels (hypoxia 15%; hyperoxia 75-40%) during developmental stages (ED 4-9) critical to avian coronary vessel development in order to alter tissue hypoxia, HIF-1a protein expression and its downstream targets. After incubation in these altered oxygen regimens, we assayed for coronary defects using anti-alpha-SMA immunohistology of sections transverse to the heart. The coronary arteries were particularly intensely labeled with anti-alpha-SMA that bound to smooth muscle cells investing these vessels. When incubated for 4.5 days under the abnormal oxygen levels, coronary arteries displayed deviations from their normal connections to the aorta. These findings indicate that developing coronary vessels may be subject to a level of regulation that is dependent on differential oxygen tension within cardiac tissues and subsequent HIF-1 regulation of gene expression. Supported in part by NIH Grants HL65314, HL0775436, ES103507.

139. Myocardial VEGF-A Regulates Coronary Vessel Development

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Coronary circulation starts soon after septation of the heart to meet oxygen demand of the rapidly growing heart when passive perfusion is no longer adequate. Abnormal coronary circulation resulting from the malformation of coronary vasculature can be deleterious to heart development. However, the mechanisms underlying coronary vessel development are poorly understood. We have begun to study the role of hypoxia and myocardial VEGF-A in coronary vessel development. To this end, we examined tissue hypoxia and VEGF-A expression in the developing mouse heart and found that VEGF-A expression is accentuated in the hypoxic myocardium at the proximal outflow tract and interventricular septum prior the formation of coronary arteries. We then deleted VEGF-A in the myocardium and observed defects in the vascular plexi and coronary ostia. These defects cause a unique myocardial necrosis restricted to the hypoxic areas where give rise to major coronary branches, leading to embryonic death around embryonic day 17.5. Further gene expression studies showed that such deletion does not alter the expression of endothelial markers, indicating that myocardial VEGF-A is dispensable for endothelial differentiation but required for the remodeling of the vascular plexi. The temporal role of myocardial VEGF-A in the remodeling of coronary vessels and ostia is currently under the investigation using a newly established inducible myocardial Cre line. Furthermore, we have engineered a genetic mouse model capable of identifying hypoxic myocardial tissues to delineate the role of hypoxic myocytes as well as hypoxia-dependent VEGF-A expression in coronary vessel development. Together, these mouse models will allow us to dissect the regulatory pathway of hypoxia-VEGF required for coronary vessel development.

140. Deletion of Nf1 in smooth muscle cells induces increased neointima formation in response to vascular injury

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Neurofibromatosis type I (NF1) is a common autosomal dominant disorder with a broad array of clinical manifestations, including benign and malignant tumors, osseous dysplasias, and characteristic cutaneous findings, among others. In addition, NF1 patients have an increased incidence of cardiovascular diseases such as congenital heart disease, cerebrovascular malformation, and hypertension. In animal models, endothelial expression of the disease gene, NF1, is critical for normal heart development. However, the pathogeneses of the more common vascular disorders are not well characterized. To examine the role of NF1 in vascular smooth muscle, we generated mice lacking the murine homolog, Nf1, in smooth muscle (Nf1smKO). These mice develop and breed normally. However, in response to vascular injury, they display a marked intimal hyperproliferation and abnormal activation of MAPK, a downstream effector of Ras. Vascular smooth muscle cells cultured from these mice also display enhanced proliferation and MAPK activity. Smooth muscle expression of the NF1 Ras-regulatory domain (GRD) rescues intimal hyperplasia in Nf1smKO mice and normalizes vascular smooth muscle cell MAPK activity in vitro. These results show a critical role for Nf1 regulation of Ras-dependent vascular smooth muscle proliferation.

141. The role of hypoxia and hypoxia inducible factor1 in regulating the Wilms' tumour suppressor gene during epicardial development

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Generation of pluripotent epicardial derived mesenchymal cells (EPDCs) via epicardial epithelium-to-mesenchyme transition (EMT) is highly important for coronary vasculogenesis. Previous reports indicate that the Wilms' tumour suppressor gene (Wt1) that is specifically expressed in the epicardium, is required for epicardial EMT. Wt1 was down-regulated upon EPDC differentiation to become components of coronary vessels. Wt1 null mice die in midgestation with an absence of epicardium, loss of subepicardial mesenchymal cells (SEMCs) and lack of large coronary vasculature. The factors regulating Wt1 expression in heart development is still not well understood especially during early steps of coronary vessel formation. Using hypoxia indicators EF5 and HIF-1α nuclear localization, we showed that the epicardium and myocardium of the atrioventricular sulcus (AVS), interventricular septum (IVS), and base of the outflow tract (OFT) are highly hypoxic and exhibit nuclear localized hypoxia inducible factor HIF-1α. Under hypoxic conditions (15%O2 for 3 days), nuclear-localized HIF1 α coincided with the pattern of Wt1-positive epicardial and subepicardial cell accumulation. Western blots showed an increase in Wt1 in the chicken embryonic heart correlated with an increase in HIF1α under hypoxic conditions. Switching of the Wt1 isoform ratio (Exon 5+/Exon5-) was significantly induced by hypoxic treatment (1% O2 for 24h) and exogenous HIF1 α over-expression in a rat epicardial/ mesothelial cell line. The Wt1 isoform with exon5 may be responsible for promoting epicardial EMT, and inhibiting apoptosis of epicardial derived cells in mammals. Hypoxia via hypoxia inducible factor 1α (HIF1α) may be a critical physiological stimulus required for Wt1 expression that in turn promotes epicardial EMT and maintains the undifferentiated state of epicardial derived cells. This is consistent with the region-dependent production and accumulation of coronary vascular progenitor cells in the early steps of coronary development.

142. Novel Mechanisms for the Stage Dependent Effects of Retinoic Acid on Cardiogenesis

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Our lab has previously observed that altering retinoic acid signaling has detrimental effects on cardiogenesis in Xenopus laevis. Excess retinoic acid caused a marked decrease in myocardial differentiation while a short treatment after gastrulation with a retinoic acid antagonist prevented the transformation of the initial myocardial sheet to a simple heart tube. In order to understand the underlying mechanisms of these changes, we have performed a microarray analysis of changes to gene expression caused by exposure to retinoic acid, a retinoic acid antagonist, or a DMSO carrier control during the brief window of time where retinoic acid is necessary for heart tube formation. RNA from at least 60, pooled embryos was used for each sample point and the experiment was performed in triplicate. The Affymetrix Xenopus laevis microarray with approximately 14,400 individual probe sets was used in the analysis. This experiment has revealed a number of novel targets or retinoic acid signaling that we have verified using whole mount in situ hybridization on whole Xenopus embryos. Many of these are not associated with the heart but were useful in demonstrating the veracity of our microarray results. Of interest to our analysis of early cardiogenesis was our discovery of multiple mechanisms for attenuating BMP signaling by retinoic acid. One of these mechanisms is an increase in follistatin expression near the developing myocardium providing evidence for this BMP antagonist in early cardiogenesis. In addition, we have discovered that retinoic acid signaling plays a role in general patterning of the lateral plate

mesoderm suggesting that some aspects of patterning of the heart may represent a subset of a larger patterning process in the lateral plate mesoderm. Overall, our study has provided several new insights into signaling events within the myocardium prior to differentiation.

143. A mouse model for VACTERL Association

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VACTERL association is the nonrandom association of multiple birth defects that include vertebral anomalies (V), anal atresia (A), cardiac defects (C), tracheo-esophageal fistua (TE), renal anomalies (R) and limb defects (L). The mechanism that underlie VACTERL association is proposed to involve perturbation of the sonic hedgehog (shh) pathway. Consistent with this model, Gli2 -/-; Gli3 +/- and Gli2 -/-; Gli3 -/- mice have been shown to exhibit the full spectrum of defects encompassing VACTERL association. We recently recovered a novel mouse mutation causing the full complement of phenotypes comprising VACTERL. This was recovered in a mouse mutagenesis screen using fetal echocardiography to identify mutants with congenital heart defects. The defect phenotypes observed in this mutant mouse model included butterfly vertebrae, imperforate anus, cardiac anomalies, tracheo-esophageal fistua, renal anomalies including dysplastic kidney, and limb defects. The cardiac defects comprise a wide spectrum of anomalies, such as dextracardia, ventricular septal defect, and outflow anomalies that include double outlet right ventricle, persistence truncus arteriosus, transposition of the great arteries, and pulmonary atresia/stenosis. The limb anomalies seen were varied and comprised syndactyly, oligodactyly or polydactyly. Surprisingly, often two or three of these limb defects were seen on different limbs of the same animal. Heritability testing and phenotyping analysis showed the mutation is homozygote recessive. Linkage analysis utilizing microsatellite DNA markers and SNPs for a full genome scan localized the mutation to a 6.0 Mb interval on mouse chromosome 19. None of the genes in this interval have a known role in the sonic hedgehog cell signaling pathway. This novel mouse model of VACTERL association suggests a monogenic origin for the nonrandom association of multiple defect phenotypes in VACTERL. Identification of the underlying mutation promises to provide new insights into regulation of the sonic hedgehog pathway and the etiology of VACTERL association.

144. Parathormone treatment after myocardial infarction in mice ameliorates late ischemic cardiomyopathy by enhanced migration of CD45+/CD34+/CXCR4+ stem cells

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Bone marrow derived stem cells improve cardiac function after myocardial infarction (MI). Recently, parathyroid hormone (PTH) was shown to regulate the stem cell niche in the bone marrow. Therefore, we analysed in a murine model of MI the influence of PTH treatment on survival, functional parameters as well as stem cell migration to the heart. Methods: 12-24 h after MI, PTH was injected daily for two weeks. 6 and 30 days after the surgical procedure, pressure volume relationships were investigated in vivo. Heart tissues were further investigated immunhistochemically and by RT-PCR. Migration was studied by cardiac FACS analysis. Results: PTH treated animals revealed a significant improvement of post MI survival. FACS data demonstrated mobilization of stem cells after PTH treatment. Histology of PTH treated hearts showed less reduction of LV wall thickness and a smaller infarct size at day 30 which was associated with an improved myocardial function. Immunohistologically, PTH treated hearts revealed an increased neovascularization of CD31+ capillaries at the borderzone which could be explained by an upregulation of VEGF-A and VEGF-receptor1 mRNA. Moreover, VEGF-A protein was mainly detected on infiltrating cells in the granulation tissue. Expression of IGF-1 receptor protein was primarily localized on cardiomyocytes (CMs) at the borderzone, which was related to a reduced number of apoptotic CMs. Migration of angiogenic CD45+/Sca-1+ and CD45+/CD34+ blood cells to the ischemic heart was increased by PTH treatment. Enhanced homing of CD45+/CD34+ stem cells was supported by a high

expression of CXCR4 (85%) and an hormone mediated upregulation of SDF-1. Conclusion: PTH application after MI ameliorates myocardial function, which may be explained by the migration of CD45+/CD34+/CXCR4+ stem cells towards an increased SDF-1 gradient in the myocardium. This may lead to improved neovascularization and cell survival via activation of the VEGF and IGF-1 pathways.

145. Changes in Fibronectin and Laminin Expression During Chick Heart Development

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During the early stages of development the myocardium differentiates into compartments occupied by cardiac myocytes and the interstitial space between these cells, which is populated by non-myocyte cell types such as cardiac fibroblasts and endothelial cells. Although some information on the structure and function of cardiac contractile proteins and the localization of membrane ion-transport proteins, as well as the pattern of corresponding genes in normal and diseased myocardial tissue is available, little is known about the role of cardiac extracellular matrix (ECM) proteins during cardiac development or in adult health and disease stages. The ECM in the adult heart and vascular wall includes both fibrous proteins and proteoglycans. Fibrous proteins are classified into two categories: structural (such as collagen and elastin) and adhesive molecules (such as laminin and fibronectin). However there is no information about the distribution and function of both fibronectin and laminin during the early stages of heart development. Fibronectin is a dimeric glycoprotein found in the ECM of most tissues and serves as a bridge between cells and the interstitial collagen meshwork. It influences diverse processes including cell growth, adhesion, migration, and wound repair. Fibronectins are major components of the ECM and blood plasma. Laminins are a large family of ECM proteins that can profoundly influence development, differentiation and disease progression. The biological effects of the laminins are mediated by surface receptors that link laminin matrices to intracellular signaling pathways. Fibronectin and laminin are made by the cardiac fibroblast. The fibroblasts are responsible in large part for production, organization, and turnover of the extracellular matrix (ECM). The effects of the ECM are primarily mediated by integrins ($\alpha_5\beta_1$ and $\alpha_v\beta_1$ for fibronectin and$\alpha_1\beta_1$, $\alpha_3\beta_1$ and $\alpha_7\beta_1$ for laminin), a family of cell surface receptors that attach the cells to the matrix and mediate mechanical and chemical signals from it. Thus we are interested in analyze the distribution and function of both fibronectin and laminin under the early stages of heart development. Using qRT-PCR we show that Fibronectin- mRNA levels were similar in both atria and ventricle from stage 13 to 40. In contrast Laminin γ1 and Laminin β1 mRNA levels were similar in both atrium and ventricle at early stages as St13 but there is an increase predominantly in the ventricle through St40. The Laminin β2-mRNA levels remains equally expressed in both atrium and ventricle during all the stages analyzed (St13-40). The integrin-subunits α5, α6 and β1 mRNA levels were expressed in both atria and ventricle; however the ventricle exhibit higher levels of expression at any other tissue (atria, Atrio-Ventricular Canal and outflow tract) at all the stages of development analyzed (St13-40). Because not always is a direct relationship between the mRNA levels and expression and function of the proteins, we performed immunostaining for fibronectin, laminin and their receptors from St13-40. In concordance with our finding of mRNA levels for fibronectin and laminin, we found that fibronectin is similarly present in atrium and ventricle at all stages of development, whereas laminin is predominately present in ventricles especially at latter stages of development. Supported by LPP-AHA-SDG 0530140N.

146. Manipulation of regulatory elements of the Tbx1 endogenous gene supports cooperative rather than modular regulation

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Pharyngeal morphogenesis is a complicated process involving precise coordination of multiple cell types and transcription networks. Tbx1, encoding a T-box transcription factor, is an important regulator of this process, thus it is critical to understand how Tbx1 is, in turn, regulated. A forkhead (Fox) transcription factor binding site was shown to be required for expression of a reporter construct in the pharyngeal

endoderm, head mesenchyme and second heart field (SHF) (Yamagishi et al., 2003; Maeda et al., 2006). We have deleted this site from the endogenous gene, but qRT-PCR experiments and in situ hybridization did not indicate a significant reduction of Tbx1 expression in homozygous embryos (Tbx1ΔFox/ΔFox). Consistently, homozygous mice were healthy and fertile. Next, we deleted a 3 Kb segment of DNA sufficient to drive expression of a reporter construct in most of Tbx1 expression domains (Brown et al., 2004). This fragment includes the Fox binding site. Homozygous mice (Tbx1ΔFG/ ΔFG) were also phenotypically normal. However, qRT-PCR revealed a significant reduction of Tbx1 expression (by approx 30%), and in situ hybridization revealed visible down-regulation in the head mesenchyme. Thus, although the Fox binding site is dispensable, the enhancer does play a role in the regulation of Tbx1, albeit a modest one. Transgenic analysis of 10 evolutionary conserved DNA segments from a 54 KB of the Tbx1 locus could not identify any segment sufficient to drive expression in Tbx1 domains, with the exception of one segment that worked as a weak pharyngeal endoderm enhancer. Overall, these results suggest that there are no discrete and robust regulatory modules within the 54 KB of the locus, but there are sequences that individually provide a small contribution to gene regulation. This arrangement may work as an "insurance policy" against mutations, and overall provide robustness to the regulatory machinery of the gene.

147. Dysregulation of Cardiogenesis, Cardiac Conduction, and Cell Cycle in Mice Lacking miRNA-1-2

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MicroRNAs (miRNAs) are a relatively new class of genes that are widely used by organisms as nodes for titrating protein translation, yet the mammalian requirement for specific miRNAs is unknown and reliable target prediction remains elusive. Targeted deletion of the highly conserved, muscle-specific, miRNA, miR-1-2 revealed several roles in cardiac development and maintenance. Partial embryonic lethality and septation defects were observed late in gestation. Loss of miR-1-2 also resulted in numerous defects in adult cardiac function. A small percentage of animals died by 3 months of age from heart failure with organized atrial/ventricular thrombi. The surviving adult animals displayed cardiac hyperplasia and cardiomyocyte karyokinesis as well as electrical conduction defects reminiscent of bundle-branch block. To better understand the rules governing miRNA:mRNA interactions we performed unbiased microarray screens of misexpressed mRNAs in miR-1-2-/- hearts, which displayed a marked enrichment for sites complementary to the miR-1 "seed match" in genes that were up- but not down-regulated. Analysis of the mRNA structure of the upregulated genes displayed a propensity of potential miR-1 binding sites for energetically accessible regions. This work reveals the necessity of proper miRNA dosage in mammals and highlights rules for improving miRNA target prediction algorithms.

148. Characterization of the Regulatory Architecture Controlling the Expression of TBX20 During Heart Development

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Mammalian cardiac development is a complex process that requires contributions from multiple distinct lineages over an extended period of morphogenesis. This process, leading to the unique structural and functional properties of the cardiac chambers, valves, and conduction system depends on the stereotyped regionalized expression of multiple transcription factors during embryogenesis . The main goal of this study is to identify the key regulatory sequences controlling the complex temporal and spatial expression patterns of TBX20. To dissect the regulatory network of Tbx20 we scanned 450,000 bp of genomic sequence that spans Tbx20 and both its telomeric and centromeric intergenic regions bracketed by the next flanking genes. We employed a combination of cross-species sequence comparisons to identify noncoding sequences in the TBX20 locus conserved over varying evolutionary distances and tested them

for their enhancer function in an in vivo mouse transgenic system. We found that 10 of the 32 groups tested displayed highly reproducible enhancer activity in domains of expression that recapitulate the endogenous Tbx20 expression at E11.5. Six of these enhancers drive reporter gene expression in discrete, yet overlapping domains of the developing heart, including compact chamber myocardium, endocardial cushions, and outflow tract. To address the critical question of the necessity of individual enhancers for the proper expression of Tbx20 a modified BAC that harbors all 6 heart enhancers driving LacZ expression is being developed. The functional dissection of these regulatory sequences will reveal the molecular basis underlying TBX20 expression and uncover new aspects of its biology in cardiac development and function.

149. Nkx-2.5 is widely expressed during induction of embryonic stem cells in monolayer culture

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Re-muscularization of damaged myocardium with embryonic stem cells (ESCs) requires induction to the cardiomyogenic lineage followed by isolation of homogeneous cardiomyocytes in numbers sufficient for transplantation. To obtain large populations of expanded cardiomyocytes, differentiation in monolayer culture is preferred to the three-dimensional environment of embryoid bodies (EBs). Because integration with host myocardium should be optimized by transplanting immature cardiomyocytes, the use of transgenic ESCs that express a fluorescent marker protein upon activation of an early cardiomyogenic promoter such as Nkx-2.5 is an attractive option. To attain these goals we evaluated the ability of transiently applied noggin to induce mouse ESCs to the cardiomyogenic lineage while monitoring Nkx-2.5 expression. Results reveal that, per Yuasa et al. (Nat. Biotechnol. 23:607;‘05), cardiomyocyte-enriched EBs are generated within two weeks of treating monolayer-cultured ESCs with noggin. During noggin treatment, the cohort of Nkx-2.5-positive ESCs was decreased, in comparison with untreated cultures in which the Nkx-2.5-positive cohort was remarkably large. Evaluation of Nkx-2.5 expression in untreated pluripotent ESCs induced to differentiate by withdrawing MEFs and LIF revealed that both must be present to maintain pluripotency, and that widespread expression of Nkx-2.5 (>90% positive cells) occurs within 16 hours of inducing differentiation. However, only ~25% of Nkx-2.5-positive ESCs express HNF-3 or brachyury, respective early markers for endodermal and mesodermal lineages. Human ESCs exhibit a similar expression pattern during transition from pluripotency to differentiation. Nkx-2.5-positive cells decline during extended growth in monolayer culture; we are now evaluating their differentiation outcomes. Because the low percentage of brachyury-positive mesoderm cells indicates that only a subset of the Nkx-2.5-expressing cells is specified to the cardiomyogenic lineage, selection of an enhancer within the modular Nkx-2.5 promoter must be carefully considered to isolate immature cardiomyogenic cells exclusive of other cell types.

150. Melanocytes in the developing and adult atrioventricular valves

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It is now established that apart from its major contribution to the outflow track, cardiac neural crest cells (CNC) reach and persist in the atrioventricular (AV) valves. We did a systematic histological analysis of the contribution of melanocytes, a subpopulation of CNC cells, in murine AV valves. Using DctLacZ mice, melanocyte precursors were identified in the embryonic cushion-like leaflets at E13.5 and persisted in AV valves at later stages. In all AV leaflets (N1.5, N4.5, 3 weeks and 8 weeks of age), differentiated melanocytes, identified by L-dopa staining, localize mostly to the atrial side and edge of leaflets. To test if melanocyte localization correlates with AV patterning of the AV valves, we analyzed the expression of the extracellular matrix (ECM) proteins Versican B and Collagen I which are found at the atrial and ventricular sides of adult AV valves, respectively. In the adult, melanocytes were found exclusively at the site of Versican B expression. To test if melanocytes could affect the AV patterning of the leaflets, ECM expression was analyzed in hypopigmented mice that lack the tyrosine kinase receptor kit, in which

melanocytes are completely absent, and in transgenic mice that over-express endothelin, in which there is an excessive number of melanocytes not only in the AV valves but also in ectopic locations such as the pulmonary valve. In these mice, however, the AV patterning of Versican B and Collagen I was not affected. These results indicate that cardiac melanocytes do not seem to affect the extracellular environment of the valves.

151. Optical imaging of cardiomyocyte action potentials with high spatiotemporal resolution

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The cardiac conduction system (CCS) orchestrates the rhythmic and coordinated depolarization of the heart. However, the electrophysiology of the CCS at various stages of embryonic development has remained unclear due to technical challenges in measuring the electrical activities with high spatiotemporal resolution. We developed an optical imaging modality to record cardiomyocyte action potentials (APs) using laser scanning confocal microscopy in conjunction with the novel fast potentiometric dye ANNINE-6, which was previously shown in cultured cells to exhibit higher voltage sensitivity than traditional dyes. Isolated, electrically paced (3 Hz), adult mouse hearts were retrogradely perfused with Tyrode's solution (20°C) supplemented with 50 μmol/L cytochalasin-D and 1 μmol/L ryanodine to effect complete electro-mechanical dissociation, and were stained with ANNINE-6. Signals were recorded from cardiomyocytes located ~20 μm below the left ventricular surface using the line-scanning mode (1042 lines/sec) of the scanning system. Each line encompassed 128 pixels (pixel area=0.04 μm2) at 3-μm z-resolution. Ensemble averaging of about 28 consecutive APs was employed to increase signal-to-noise ratio (SNR). Signal processing was applied to further improve the SNR without signal averaging. Using laser illumination (488 nm) through a 40x1.2NA water immersion objective, ANNINE-6 exhibited decreases in fractional fluorescence, ΔF/F, per AP, in the red and infrared emission range (>560 nm; peak ΔF/F= -23 ± 4.7% per AP). APs were resolved from side-to-side and end-to-end connections of neighboring cardiomyocytes as well as from single or multiple t-tubules of individual cells. Thus, the high voltage sensitivity of ANNINE-6 opens the opportunity to record APs from intact hearts on a subcellular to multicellular scale. This feature bodes well for high-resolution electrophysiological characterization of the developing murine CCS.

152. Effects of retinoic acid and TGFβ2 signaling on fibronectin expression and proepicardial-derived cell behavior during epicardium development

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The epicardium is derived from the proepicardium (PE), a group of mesothelial cells that develops during embryonic day (E) 9.0 in the mouse. By E10-10.5 the proepicardium to epicardium transition is nearly complete and the heart is fully covered with epicardium by E11. Abnormalities in the epicardium lead to cardiac defects as shown in the retinoid X receptor alpha knockout (RXRα-/-) mouse, a model of congenital heart disease that exhibits defects in various regions of the heart. We have shown previously that transforming growth factor beta2 (TGFβ2) is elevated at midgestation in the RXRα-/- and we now show that it also appears elevated in the PE of the RXRα-/-, indicating a possible role for TGFβ2 in the RXRα-/- epicardium malformation. Our lab has also shown that the extracellular matrix protein fibronectin (FN) is elevated during epicardium formation in the null. PE explants from RXRα-/- show increased and disorganized FN and alterations in migration of PE-derived cells. Here we show that TGFβ2 treatment of PE explants promotes activated Smad2 (pS2) nuclear localization while co-addition of 9-cis retinoic acid (9-cisRA) with TGFβ2 enhances pS2 nuclear localization. TGFβ2 treatment also increases FN levels, indicating a possible mechanism by which RXRα-/- displays increased FN. In contrast, treatment with 9-cisRA alone or together with TGFβ2 decreases FN. Treating explants from embryos 23 somites or less (<23) with TGFβ2 decreased migration of PE-derived cells and increased FN while it has little effect at greater than 24 somites (>24). Treating with TGFβ2 and 9-cisRA at <23

somites restores migration and normalizes FN levels while at >24 somites it appears to cause a decrease in migration with no effect on FN levels. We hypothesize that retinoic acid regulates TGFβ2-induced FN expression and that altered retinoic acid signaling disrupts TGFβ2 regulation and results in abnormal epicardium development.

153. Trichloroethylene Regulation of Calcium Signaling Pathways And Reduced Cardiac Function In Vitro

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Our laboratories have been investigating the molecular mechanisms by which trichloroethylene (TCE) may induce cardiac malformations in the embryonic heart. We hypothesize that study of changes of gene expression induced by TCE will identify critical cellular pathways altered by this toxicant during crucial phases of cardiac differentiation. We began by investigating specific gene expression changes produced by exposure of embryo-derived cells to TCE. Microarray analysis indicated that TCE disrupted the expression of genes encoding for proteins involved in regulation of calcium fluxes in cardiac cells, including Ryr, Serca2a and Camk. Changes in expression levels of these genes were confirmed by real-time PCR. These results indicate a dose- and time-dependent pattern of regulation at the transcript level. In particular, we observed a bi-modal effect of low (1-100ppb) and high (1-10ppm) doses of TCE on mRNA expression. As disruption of intracellular calcium pathways could explain perturbation of normal heart development, cell imaging was used to characterize the effects of TCE on regulation of calcium fluxes in rat cardiac H9c2 myocytes. The data show significant changes in intracellular calcium fluxes in the treated cells. The pattern of changes included slower time to peak calcium, lower peak calcium and slower recovery after stimulation with vasopressin. Thus, TCE appears to alter genes crucial for embryonic development, particularly genes involved in calcium signaling pathways. Cell imaging analysis confirmed that exposure to TCE disrupted the ability of myocytes to regulate cellular calcium fluxes. This perturbation of calcium may disrupt both cardiac physiology and intracellular signaling and suggests likely mechanisms for TCE-mediated cardio-toxicity.

154. Depletion of cardiac myosin light chains affects cardiac function, sarcomere assembly and cardiomyocyte cell size in zebrafish

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Myosin light chain (MLC) comprises two subfamilies, the essential light chains (ELCs) and the regulatory light chains (RLCs). Although mutations in both gene subfamilies have been linked to human hypertrophic cardiomyopathy, their functions during cardiogenesis in a vertebrate animal are not well understood. We have characterized the expression of 12 zebrafish MLC homologues and identified cmlc1 as a single ELC orthologue, and cmlc2 as a single RLC orthologue that exhibit cardiac-specific expression during zebrafish embryogenesis. Reduction of either cmlc1 or cmlc2 using morpholino-modified antisense oligonucleotides leads to compromised cardiac function, disrupted assembly of A-bands and Z-discs, but not the assembly of the nascent striated myofibrils including the thick filaments. Interestingly, reduction of one gene leads to the activation of the other; however, the two genes are not functionally redundant, as injection of the mRNA of one gene cannot rescue the morphant phenotype of the other. Furthermore, our data suggest that reduction of the two genes might differentially affects cardiomyocyte size, and sequentially the size of ventricular chamber. In summary, our data provide insights on distinct functions of MLC and ELC during cardiogenesis and possibly hypertrophic cardiomyopathy.

155. NFATc1 in endocardial cushion elongation and remodeling

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While it is known that Nuclear Factor of Activated T-cells variant c1 (NFATc1) is necessary for the elongation and remodeling of endocardial cushions (ECs) into mature cardiac valve leaflets, the molecular mechanisms by which NFATc1 promotes these processes have not been elucidated. EC elongation and remodeling is marked by increased cell proliferation and changes in extracellular matrix (ECM) architecture. During this time, NFATc1, Vascular Endothelial Growth Factor-A (VEGF), and Receptor Activator of NFkB Ligand (RANKL) expression is localized to ECs. Experiments with adult human pulmonary valve endothelial cells show that VEGF treatment induces cell proliferation via an NFATc1 dependent mechanism, while our data demonstrate that the RANKL/NFATc1 target gene Cathepsin K (CtsK) is expressed in endothelial cells of elongating and remodeling murine ECs. Our hypothesis is that NFATc1 activation in EC endothelial cells via VEGF or RANKL signaling promotes EC endothelial cell proliferation and production of ECM remodeling enzymes respectively. In order to determine the effects of VEGF and RANKL signaling on cells in elongating and remodeling valves, chicken embryo ECs are explanted to collagen coated chamber slides and maintained in culture for two to six days. Addition of VEGF causes increased NFATc1 nuclear localization, high expression of NFATc1 in mitotic cells and increased proliferation. RANKL treatment of cultured ECs increases transcription of the NFATc1 target gene CtsK. CtsK is an ECM remodeling enzyme normally expressed in elongating and remodeling EC endothelial cells, but absent in NFATc1-/- mice. Studies are in progress in NFATc1 mutant mice to determine if lack of NFATc1 results in reduced EC cell proliferation and lack of additional ECM remodeling enzymes in vivo. Our results are consistent with VEGF activation of NFATc1 to promote EC cell proliferation, and RANKL activation of NFATc1 to promote ECM remodeling enzyme production.

156. Conditional Ablation of a Subset of Cells of the Proepicardium

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The coronary vasculature is generated from a population of progenitor cells that originate from the proepicardium (PE). The PE is a small "grape-like" transient organ that gives rise to both the epicardium (outer layer the heart) and to the vasculature of the heart. We have begun to examine the physiological consequence of the removal of a subset of cells of the proepicardium using a cell-specific ablator transgenic mouse line (pudeltatk), and a transgenic mouse line that expresses cre recombinase under the control of various promoter elements from the SRF gene (4xPEmCArG). Preliminary analysis using Rosa26R animals has shown that the 4xPEmCArG construct in E10.5 mouse embryos is expressed in subsets of cells in the proepicardium as well as in other tissues. In neonates, the expression of the construct is found in the cells of the coronary vasculature. To begin to study the functional role of this subset of proepicardial cells we mated the 4XPEmCArG Cre mouse line to a pudeltatk mouse line. The pudeltatk line contains a LoxP interrupted modified herpes simplex virus thymidine kinase gene that is only expressed in cells after cre mediated recombination. Upon the administration of the prodrug Ganciclovir (GCV) proliferating cells expressing the pudeltatk gene will be ablated. Pregnant mice were given daily IP injections of GCV (100mg/kg) starting at E10.5, and embryos were harvested at stages E11.5, E12.5 and E13.5. Mutant E13.5 embryos displayed phenotypic variations particularly in the severity of the phenotype. Some embryos appeared to have smaller jaws and the majority of the mutant embryos had varying degrees of blood hemorrhaging and peripheral vascular defects. E11.5 and E12.5 mutant embryos did not exhibit consistent phenotypes suggesting that the effect of the cell ablation may not be immediately visible in early embryos or that the study is underpowered.

157. Tgfbr3 null mice have increased atrioventricular cushion volume

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Transforming Growth Factor β (TGFβ) regulates endocardial cell epithelial mesenchymal transformation (EMT) in vitro and valvular remodeling during development in vivo. Two classes of receptors bind TGFβ. The first class consists of two serine/threonine kinase receptors termed Type I (TGFBR1) and Type II (TGFBR2). The second class contains two transmembrane proteins termed the Type III TGFβ receptor (TGFBR3), or betaglycan, and endoglin. TGFBR3 binds all TGFβ ligands with similar affinity and can present ligand to the TGFBR2/TGFBR1 complex. Gain- and loss-of-function experiments have demonstrated a requirement for TGFBR3 in TGFβ-stimulated EMT, a necessary step in heart valve formation. Targeted deletion of Tgfbr3 in the mouse results in a spectrum of cardiovascular defects that include overriding aorta or double outlet right ventricle, VSD, and a thinned myocardium. Embryos die at E14.5 due to failed coronary vessel development. Given that mice null for Tgfb2, the ligand that binds uniquely TGFBR3, have valvular hyperplasia, we asked if Tgfbr3 null mice have a similar phenotype. We analyzed the atrioventricular cushion volumes of E13.5 littermate pairs by analysis of serial sections using MetaMorph®. No significant difference was seen between wild type 0.10 ± .006 mm3 and heterozygous null embryos 0.10 ± .005 mm3 (n=3; $P>0.8$). However, comparison of null embryos to wild type or heterozygous null embryos revealed a significant increase in volume in nulls. We observed an average AV cushion volume of 0.10 ± .006 mm3 (n=5) in control animals as compared to 0.15 ± .01 mm3 (n=5) in null embryos ($P<0.05$). Therefore, Tgfbr3 deletion results in valvular hypertrophy. Supported by HL52922, HL67105, and HL083811.

158. Changes in Sodium Channels Expression Levels During Cardiogenesis in a Transgenic Mouse Model of Long-QT Syndrome

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The embryonic heart initiates an asynchronous and peristaltic contraction but during subsequent development acquires a synchronous contraction coordinated through a specialized tissue the cardiac conduction system. The voltage-gated sodium channels (formed by subunits alpha and beta) are the responsible of the fast increase of the permeability of the cellular membrane to Na+ that happens in the initial phase of the action potential in cardiomyocytes, but the possible modulation of the sodium currents by the β subunits in cardiomyocites continues being controverter. Recently we have described the expression patterns of the alpha subunit of the cardiac sodium channel (Scn5a) and the beta subunit (Scn1b) during cardiac development, demonstrating that both are preferential expressed in the components of the ventricular conduction system. By other hand, the morphologic and molecular characterization of a mouse model of long QT syndrome (α -MHC-KvLQT1-iso2-T7) carried out in our laboratory revealed the presence of a dilated and hypertrophy cardiomiopathy along with the arritmogenic pathology. In addition, we have detected Scn5a and Scn1b overexpression in adult heart carried α -MHC-KvLQT1-iso2-T7 transgene. To determine if the dilated and hypertrophy cardiomiopathy in this mouse model is secondary to the Scn5a and Scn1b overexpression, or vice versa; we have analysed the cardiac morphology as well as expression patterns of Scn5a and Scn1b throughout the embryonic development in this transgenic model. In agreement with the results obtained until the moment, an increase of Scn5a and Scn1b expression levels were observed between E13.5- E14.5, while morphologic sings of dilated and hypertrophy cardiomiopathy no were detected until E18.5. This study can have important repercussions in the understanding of the mechanisms and consequences of the electrophysiological alterations in new born and can open new ways to better understanding of clinical disorders such as congenital anomalies, arrhythmias and perinatal sudden death.

159. Fate mapping regions of the posterior Second Heart Lineage in the mouse

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The mesodermal cells that form the early heart are derived from two populations, the First and Second Heart Lineage (FHL & SHL). The FHL, derived from the lateral plate mesoderm, will forms the cardiac crescent at about E7.5 in mouse embryos. The SHL lies dorsal and, in parts, medial to the crescent, localized in the pharyngeal mesoderm and is defined by islet1 expression pattern. In summary, the left ventricle (LV) is exclusively derived from the FHL whereas the distal outflow tract (OFT) is a product of the SHL. The proximal OFT and right ventricle (RV) are predominantly SHL derived, and the atria are of mixed F&SHL origin. A sub-portion of the SHL expresses FGF10, as shown by the 1v-24 lacZ reporter, which labels the so-called Anterior Heart Field (AHF). The AHF is fated to contribute to the OFT/RV but not the inflow/atria. Presumably, the islet1+/1v-24- portion of the SHL contributes to inflow only. Using dye labelling we are fate-mapping the SHL to explore its contribution to the developing heart. At 2-3 somites, the posterior portion of the AHF contributes to the proximal portion of the OFT and RV. The contribution to the different regions of the OFT (i.e. circumferentially) are being analyzing at this moment. Both left and right "posterior heart field" (PHF) contributes to left and right common atrium, respectively. Further stages (4-6 somites) the contribution of the PHF to the left and right component of the atrium remains unaltered in respect to previous stages. However, it appears that the left PHF contributes to the atrioventricular canal (AVC) (superior region) more than the right. At these stages the contribution to the OFT give rise to the distal portion in all cases. These observations will be correlate with spatial and temporal gene expression, particularly of the nodal-pitx2 pathway.

160. Furin is necessary for proper heart remodelling

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Furin is an endoprotease of the family of mammalian proprotein convertases and is involved in the activation of a large variety of regulatory proteins by cleavage at basic motifs. Furin is required for ventral closure and axial rotation, and formation of the yolk sac vasculature. Moreover, furin activity is essential in cardiogenic mesoderm to promote heart looping. We studied the cardiac expression pattern of furin mRNA from E9.5 to birth and showed that its expressed in cardiac and mesenchymal cells of the heart. Deletion of furin gene in whole embryo is lethal early in development and hence preclude the study of its function later in cardiac development. In order to overcome this drawback, we inactivated the furflox allele conditionally in the heart. Furflox/flox mice were crossed with xMlc2-cre transgenic mice. In this transgenic line, a 3 kb fragment of the Xenopus laevis myosin light-chain 2 (xMLC2) promoter drives Cre recombinase expression in a cardiac-restricted fashion in the mouse embryo. Less than 5% of xMlc2-cre; furflox/flox homozygous embryos live to adulthood. Mendelian ratio is normal until E18.5 but drop dramatically at birth indicating that mice are dying around parturition. High Resolution Episopic Microscopy study on E18.5 mutant hearts indicates that ventricular septal defect are present in about 50% of xMlc2-cre; furflox/flox.

161. PDGF-BB Rescues Coronary Vessel Formation in FOG-2 Deficient Hearts

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Formation of the coronary vessels is a complex, essential component of heart development. Disruption of coronary vessel formation has deleterious effects on both heart development and function. While much is known about the cellular movements involved in the de novo formation and subsequent remodeling of the coronary plexus, the molecular regulators of this process are less well understood. Recent studies have highlighted the importance of epicardial-myocardial communication in the regulation of coronary vessel development. Previous work has demonstrated that myocardial expression of the transcriptional co-

repressor FOG-2 is essential for coronary vessel development. Mice lacking FOG-2 fail to form a complete coronary vascular plexus and this phenotype can be rescued by myocardial expression of FOG-2. To deepen our understanding of FOG-2's role in the regulation of coronary development, we examined the expression of genes known to be involved in coronary development in FOG-2 deficient mice. Using a combination of microarray analysis, quantitative RT-PCR and in situ hybridization, we identified two signaling molecules, Sonic Hedgehog (Shh) and Platelet Derived Growth Factor B (PDGF-B), whose expression is significantly down-regulated in FOG-2 deficient hearts. To demonstrate the importance of these factors for coronary plexus formation, we used an in vitro organ culture system to perform coronary rescue assays on FOG-2-/- embryonic hearts. In these assays, PDGF-BB protein was able to restore coronary plexus formation in embryonic hearts lacking FOG-2. These results suggest that PDGF-B is downstream of FOG-2 in a molecular pathway that regulates the development of the coronary vascular plexus.

162. Arrhythmias Cause Embryonic Lethality in Tbx3 Mutant Mice

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The transcription factor, TBX3, is mutated in human Ulnar-Mammary Syndrome. The molecular bases of the complex human phenotypes are not understood. Tbx3 homozygous null mutant mice die in mid-gestation with mammary gland, limb, and yolk sac defects. Tbx3 is also expressed in the conduction system of the developing heart. We generated a series of Tbx3 hypomorphic alleles to examine the dosage-dependent requirements for Tbx3 during mouse development. The Tbx3GH allele contains a hygromycin resistance gene 3' to the Tbx3 locus; the hygr gene can be removed with flp to generate the Tbx3G allele. The most severe hypomorphs are compound heterozygotes (Tbx3 GH /null) which die by e12.5 and have limb defects that are indistinguishable from those in null homozygotes. Tbx3G/G embryos display a less severe hypomorphic phenotype; they survive to at least e13.5 with mildly affected limbs. Given the potential importance of Tbx3 in conduction system development, we interrogated heart rate, rhythm and contractility by ultrasound on e10.5 to e13.5 embryos in utero. Cardiac morphology was also assessed histologically. Tbx3GH/null embryos are indistinguishable from controls at e10.5 but can identified at e11.5-12.5 by 2:1 or higher grade AV nodal block. Some mutants display poorly coordinated ventricular contraction consistent with dysfunction of the His-Purkinje system. Tbx3G/G hypomorphs also develop arrhythmias, but later in development. Atrial rate and contractility are preserved until immediately premortem. These functional defects are associated with abnormal development of the atrioventricular canal and ventricular septum. Our data support a dosage-dependent requirement for Tbx3 in multiple aspects of cardiac morphogenesis and function. We are currently investigating whether cell specification, organization, and electrical coupling of the specialized conduction system tissues are affected by Tbx3 deficiency.

163. Paracrine signals promote embryonic stem cell-derived cardiomyocyte specification

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Using current methods cardiogenesis in embryonic stem cells is an inefficient process, with only a small percent of cells differentiating to cardiomyocytes. Thus, the further identification of growth factors and signaling cascades that influence cardiomyocyte specification from embryonic stem cells would advance the development of efficient strategies to generate homogenous populations of embryonic stem cell-derived cardiomyocytes and may reveal new paradigms for the treatment of cardiovascular disease. Paracrine signals derived from mouse visceral endoderm-like (END2) cells have also been shown to promote cardiogenesis in embryonic stem cell cultures. Whereas Wnt antagonists and Nodal are known to regulate the secretion of endoderm-derived factors with heart-inducing activity in Xenopus, the endoderm-derived signals that promote embryonic stem cell cardiogenesis have remained largely uncharacterized. Thus, to identify candidate heart-inducing molecules, we developed inductive co-culture assays and cell mixing experiments using mouse embryonic stem cells. We examined the developmental

step stimulated by END2-derived factor(s) as well as temporal competence to respond to the endoderm-derived inductive factor(s) and demonstrate that END2-derived factor(s) act at the point of stimulating competent mesendoderm (brachyury+) to enter the cardiac lineage (Nkx2.5+). Interestingly, constitutive activation of TGFb Nodal signaling in END2 cells significantly increases cardiomyocyte differentiation in mouse embryonic stem cells and cell mixing experiments further indicate that the Nodal signaling pathway controls the production of a diffusible factor that acts cell non-autonomously to induce cardiogenesis. Studies with mutant embryonic stem cells show that neither Nodal nor a shed version of Cripto, as has been proposed previously, is responsible for the cell non-autonomous activity. Taken together, these data suggest an evolutionarily conserved pathway involving endodermal cells mediates murine heart induction.

164. Conditional Deletion of Focal Adhesion Kinase Leads to Defects in Ventricular Septation and Outflow Tract Alignment

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To examine a role for FAK in cardiac morphogenesis, we generated a line of mice with embryonic conditional deletion of FAK in nkx2-5-expressing cells. Mice with conditional FAK-deficiency (termed FAKnk) died shortly after birth due to cyanosis likely due to a profound sub-aortic ventricular septal defect (VSD) and associated mis-alignment of the outflow tract, which was apparent in 89% of FAKnk embryos. No significant differences were observed in proliferation or apoptosis between control and FAKnk hearts. However, decreased myocardialization was observed in the conal ridges of the proximal outflow tract in FAKnk hearts, indicating that the defects observed may result from impaired cell migration. Interestingly, chemotaxis was significantly attenuated in isolated FAK-null cardiomyocytes in comparison to genetic controls and these effects were concomitant with reduced tyrosine phosphorylation of p130CAS. Thus, it is possible that ventricular septation and appropriate outflow tract alignment is dependent upon FAK-dependent p130CAS activation and subsequent induction of polarized myocyte movement into the conal ridges. However, since FAK was deleted from additional non-myocyte cells in the FAKnk hearts (including derivatives of the anterior heart field, endoderm, and pharyngeal arch ectoderm), future studies will be necessary to determine the contributions of these various lineages to the phenotypes observed.

165. TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) decreases extra-embryonic vasculature in the early chick embryo

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Injection of chicken eggs with the environmental pollutant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) reduced and altered vessels in area vasculosa. TCDD is a chlorinated aromatic compound with documented adverse effects on the developing cardiovascular system. It is widespread in the environment, very resistant to degradation and highly lipophilic, leading to its concentration in fatty tissues. Humans are exposed mainly through food of animal origin. TCDD has high affinity for and exerts its toxic activity through binding to the aryl hydrocarbon receptor (AhR). Ligand-bound receptor dimerises with the AhR nuclear translocator (Arnt) and acts as a transcription factor on genes carrying the xenobiotic response element in their promoter. We injected chicken eggs with 3.5 ng TCDD/g egg dissolved in an emulsion of peanut oil, lecithin and water. Controls received emulsion only. Two µl of fluid was injected into the sub-germinal space at approximately the three somite stage and the eggs were then incubated for another 48 hours. After this early exposure to TCDD, at HH17-19, the embryos displayed overall normal bodies and had a beating heart that circulated blood through the body. However, in general the vessels in area

vasculosa were fewer and thinner and not as many had developed into major arteries and veins of increased thickness and length, as compared to the outcome in controls. Moreover, vessels in exposed embryos showed an alteration in the smooth outward growth towards sinus terminals. Instead of running straight between branches their direction often changed randomly. These preliminary results suggest an effect of TCDD on the angiogenetic development and remodelling of the primary capillary plexus. Communication between cells leading to some vessels increasing in size and also the orientation of vessels seems to be disturbed.

166. Jagged1-Notch signaling from endothelial cells is required for cardiovascular development and vascular smooth muscle differentiation

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In humans, mutations in the Notch ligand Jagged1 result in Alagille syndrome, an autosomal dominant disorder that is characterized in part by congenital heart disease affecting the cardiac outflow tract. However, the mechanism by which Jagged1-Notch signaling influences cardiac development remains poorly understood. In particular, cardiac outflow tract development involves complex interactions between several cell types, including endothelial cells, cardiac neural crest cells, and cardiac myocytes. However, there have been a limited number of tissue-specific studies to address the role of Jagged1 or Notch in these different cell types. We have shown that inhibition of Notch signaling in the cardiac neural crest in mice results in congenital heart defects that mimic those seen in Alagille syndrome, including pulmonary artery stenosis, ventricular septal defects, and aortic arch patterning defects. These findings are associated with failure of neural crest progenitors to differentiate effectively into vascular smooth muscle. Furthermore, we show that endothelial-specific deletion of Jagged1 in mice results in embryonic lethality and severe defects in vascular smooth muscle development. Together, these results suggest that Jagged1 expressed on endothelial cells signals to Notch receptors on adjacent neural crest cells to promote vascular smooth muscle differentiation and normal cardiovascular development. This provides a model to explain a role for Jagged1-Notch signaling in the pathophysiology of congenital heart disease.

167. To generate cardiac mutants in zebrafish using a gene-breaking transposon cassette

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Zebrafish is becoming a useful vertebrate model organism for studying the cardiovascular system. Forward genetic screens in zebrafish using either ethyl nitrosourea (ENU) or pseudotyped retrovirus as mutagens have led to the generation of about 100 mutants with cardiac defects, which provide insights on molecular mechanisms of cardiogenesis at whole-genome level. Here we report a novel forward genetic screening strategy to systematically generate cardiac mutants using gene-breaking transposon as a mutagen. From 220 F0 founder fish, we have generated 55 GFP positive F1 families, which sequentially led to the identification of 38 embryonic lethal mutants in 33 F1 families. 15 out of the 38 mutants affect either heart formation or cardiac function. We are in the process of cloning the mutated genes. Our data demonstrated that gene-breaking transposon cassette is an ideal mutagenesis approach with high mutagenicity rate, which can be applied in a small lab to systematically study molecular mechanisms underlying cardiogenesis and the establishment of cardiac function.

168. A possible role of tenascin-C in coronary arteries in mouse embryonic hearts

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Tenascin-C(TNC) is an extracellular matrix glycoprotein transiently expressed at restricted sites associated with cell migration and mesenchymal-epithelial transformation during cancer invasion, wound healing and embryonic development. It has strong bioactivity and is suggested to be involved in neovascularization in adult tissues. To elucidate the role of TNC in coronary vasculo/angiongenesis, first, we analyzed the development of coronary vessels of from ED 9.5 to 14 mouse embryonic heart by immunostaining for PECAM, alpha-smooth muscle actin and tomato lectin staining and compared with expression of TNC using transgenic mice in which lacZ-staining reflects expression pattern of TNC. Coronary vessel progenitor cells transfer to the surface of primitive heart tube on ED9.5 from the proepicardial organ (PEO), migrate over the heart surface of to form epicardium. On ED12, cells migrated into myocardium to form vascular like structures expressing PECAM, which gradually develop along outflow tract, reached at the aorta on ED13.5. Initially, multiple PECAM positive strands were connected to the aorta, eventually fused to form a single tube of proximal part of right and left coronary arteries, respectively. Although strong expression of TNC was observed in PEO, it was quickly downregulated when cells moved on to the heart, and not detected during cell migration to form the epicardium. TNC became detectable at the developing front of PECAM positive primitive vascular channel. Next, we raised a monoclonal anti-TNC antibody and administered to the embryos through placenta at ED 13, when multiple premature coronary arteries are being to organized to a single channel. Although the hearts developed without severe anomaly, the antibody treatment significantly increased incidence of double orifices of coronary arteries. The results suggest that TNC might play a critical role in maturation of coronary vessels.

169. Understanding overlapping roles of Tbx5 and Nkx2-5 in the cardiac programme

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Congenital heart defects (CHD) are the clinical manifestation of anomalies in heart development. This development is a complex process requiring the precise integration of cell type-specific gene expression and morphological development; both are intertwined in their regulation by transcription factors. The T-box transcription factor gene TBX5 and the homeodomain gene NKX2-5 can cause inherited CHDs if they are mutated in humans. It was shown that TBX5 and NKX2-5 physically interact and converge on common downstream targets during heart development and synergistically promote cardiomyocyte differentiation. It is not know however to what degree these factors overlap in their regulation of cardiac gene expression. We used a genetic approach to generate Nkx2-5lacZ/lacZ ;Tbx5del/del knockout ES cell lines. Using tetraploid complementation we generated double knockout (DKO) mutant embryos. Our initial experiments showed a very severe phenotype in DKOs in compare to the morphological phenotype of each individual mutation revealing some initiation of cardiac differentiation in the DKO mice where the heart tube fails to form and expand. These results suggest that Tbx5 and Nkx2-5 have important overlapping roles in regulating early events in heart formation.

170. BMP type II receptor is required for valve formation and septation in the atrioventricular canal

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Homozygous mice carrying a hypomorphic mutation for the BMP type II receptor (KO) have defective septation of the outflow tract and absent semilunar valves, and die between E11.5 and birth. Here, we analyzed the atrioventricular (AV) canal and ventricles during the development of KO mice from E10.5 to E13.5. In wild-type (WT) embryos, the inferior (IC) and superior cushions (SC) were present in the AV canal at E10.5 and were fused together and to the mesenchymal cap of the atrial septum at E12.5. At E13.5, septation of the heart was completed by fusion to the ventricular septum. In KO, the SC was hypocellular at E10.5. At E13.5, the IC and SC were not fused together, to the mesenchymal cap of the atrial septum, or to the ventricular septum, resulting in atrial and ventricular septal defects. In WT, the lateral cushions appeared at E12.5 and were remodeled, along with the fused IC and SC, into the mesenchymal leaflets of the AV valves at E13.5. In KO, however, the lateral cushions were absent at E12.5 and the fused IC and SC did not shape into leaflets, resulting in AV valve defects. Defects in the myocardium were also observed in the KO at all time-points analyzed, including loose myocardium in the AV canal and ventricular septum, defective trabeculae, and thinner compact myocardium. Finally, the subepicardial mesenchyme, which provides the precursors of the coronary vessels, developed in WT from E10.5 onwards. In the AV sulcus of KO, the subepicardial mesenchyme was hypocellular and enlarged coronary vessels were observed at E13.5. Therefore, while some of the defects observed in KO mice recapitulated the defects seen in mice with deletion of BMP ligands or BMP type I receptors, others were unique to the KO indicating previously unforeseen roles for BMP signaling during heart development.

171. Colocalization of Active TGF-beta, Hyaluronan and RHAMM During Cardiac Cushion Morphogenesis

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Cardiac septation and valvulogenesis begin when endocardial cells in the atrio-ventricular junction and outflow regions undergo an epithelial-to-mesenchymal transition (EMT), and invade the underlying cardiac jelly. Members of the TGF-beta family are key regulators of this process. Hyaluronan (HA) has also been shown to be crucial for EMT. In addition to its structural role, HA promotes signaling events that are critical in the formation of endocardial cushions. The receptor that HA utilizes for these events remains unknown. We therefore examined the expression of HA and RHAMM (Receptor for Hyaluronan-Mediated Motility) during mouse endocardial cushion development and correlated this with TGF-beta signaling, localized using transgenic mice expressing a TGF-beta-responsive promoter driving GFP. During endocardial cushion formation (e9.5-e10.5), active TGF-beta and RHAMM were expressed in both the myocardium and the endocardium. Double staining for RHAMM and HA revealed that HA was concentrated around RHAMM-expressing invading cells. Later in gestation (e12.5-e13.5), RHAMM was predominately expressed in the myocardium. During EMT, the 70 kD cell surface isoform of RHAMM predominated, whereas later in gestation, the 95 kD, intracellular form was seen. RT-PCR has revealed the presence of a single, full length RHAMM transcript during the period of EMT, and no evidence of alternatively spliced transcripts or of alternate transcriptional start sites was found. Anti-TGF-beta antibody treatment of migrating cushion cells was associated with a switch from the 70kDa to the 95kDa form of RHAMM. We conclude that active TGF-beta, RHAMM and HA colocalize during endocardial cushion formation. TGF-beta regulates the expression of cell surface RHAMM, and this form may therefore mediate HA-induced EMT. As cardiac development proceeds, a transition to the intracellular form of RHAMM is seen. These alternate isoforms of RHAMM appear to arise from a single transcript and may result from alternate translation start sites or from protein processing.

172. Regulation of NFATc1 Expression Identifies a Unique Subpopulation of Endocardial Endothelium in vivo and in vitro

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Heart development begins with the assembly of a simple heart tube consisting of two interacting layers, the outer myocardium and inner endocardium. While the myocardium's role in cardiac morphogenic events has been well documented, the function of the early endocardium, its interaction with the myocardium, and how endocardial precursors are distinguished from other endothelial populations remains unclear. As one of the few specific markers of the early endocardium in murine heart development, the transcription factor Nuclear Factor of Activated T-Cells (NFATc1) is expressed in endocardial precursors as they differentiate from the cardiac mesoderm, and throughout all the endocardium of the E8.5 heart tube, prior to being restricted to the endocardial cells of the atrioventricular canal (AVC) and outflow tract (OFT). Utilizing BAC recombineering to characterize elements of the NFATc1 locus, we are now able to recapitulate expression in the early endocardium, providing us with a novel tool in which to monitor and manipulate this cell population during heart development. Furthermore, we have documented endocardial differentiation, defined by NFATc1 expression, in vitro with embryonic stem cell differentiation into embryoid bodies. In this model system, NFATc1's expression in embryoid body differentiation mirrors its in vivo pattern, upregulated at the same time as other early vascular markers while preceding cardiomyocyte differentiation. In addition, an immunofluorescence study in later embryoid bodies reveals NFATc1 positive/PECAM positive endothelial cells form a scaffold surrounding differentiating cardiomyocyte foci. Ongoing experiments are focused on introducing a fluorescent reporter and a suicide gene under control of the NFATc1 BAC into ES cells to track endocardial cell differentiation and to determine how loss of endocardial cells at various stages of differentiation effects myocardial differentiation. This system will provide the opportunity to clarify the nature of endocardial-myocardial interactions in heart development.

173. Tissue-specific Alternatively Spliced Protein 4.1R Isoforms in the Heart of the Zebrafish, Danio rerio.

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Introduction: Protein 4.1R (4.1R) has been identified as the major component of the erythrocyte membrane cytoskeleton involved in maintaining the erythrocyte shape and controlling membrane mechanical properties. In the zebrafish heart different isoforms are localized in the ventricle and atrium respectively identified following the method of immunohistochemistry. Objectives and methods: To identify the 4.1R isoforms, several cDNAs were isolated using RT-PCR. Results: Full length cDNA encoding zebrafish p4.1R (ZF4.1R) (NM_175084) is composed of putative functional domains that correspond to the human 4.1R N-terminal 30kDa FERM (four-one, ezrin, radixin moesin) domain, a spectrin and actin binding domain (SABD) and a C-terminal domain (CTD), two unique domains (UD) and an amino acid repeated domain. Four DNA fragments from heart sample were amplified. Heart2G12 was encoded the entire FERM domain plus truncated UD and CTD. Of note, the Heart3 encoded only truncated UD and whole SABD and CTD. Conclusion: In the ventricle, the Heart 2 gene products may compensate each other for maintaining the cell shape and its physiological function. The Heart3 gene product may be involved in stabilizing cardiomyocyte structure in the atrium. This is suggested because the hetero dimmer of spectrin is important in stabilizing the plasma membrane and only SABD in the human 4.1R molecule is essential to bind with hetero dimmer of spectrin. These results supported our hypothesis that in the zebrafish, each tissue-specific alternatively spliced gene product of 4.1R will have different functions in different tissues.

174. Generation and Evaluation of Tissue Specific Conditional Null Jumonji Mice

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The jumonji (jmj) gene has been shown to play important roles in cardiovascular development, the neural tube fusion process, and hematopoiesis in mouse embryos. We have previously reported that knockout of jmj (jmj -/-) results in heart malformations, which mimic human congenital heart diseases. These malformations include ventricular septal defect (VSD), thin ventricular wall, and double outlet right ventricle (DORV). All jmj -/- mice die before or around birth, depending on the genetic background of the mouse. Although the importance of jumonji in proper cardiac development is evident and the molecular roles of jumonji are beginning to emerge, it is important to examine the tissue and cell lineage specific roles of jumonji in the heart. Therefore, in this study we employed Cre-loxP technology to generate myocardial (using a myosin heavy chain-Cre, or Nkx2.5-Cre, jmjmyo), and endocardial (Tie2-Cre, jmjen) specific jumonji mutants. The jmjmyo mice exhibit a normal Mendelian ratio and appear normal upon examination. Interestingly, the jmjen mice display a significantly lower than normal Mendelian ratio at all time points examined (6.9% versus the expected 25%). Further, examination of jmjen mice indicates that they recapitulate the cardiac defects of the jmj -/- mice exhibiting both VSD and DORV. The Tie2 gene encodes an angiopoietin receptor and is detected as the first endothelial cells arise, and remains positive in endothelial cells throughout development. In mice, at E 9.5-10, a subset of cardiac endothelial cells in the atrioventricular canal and outflow tract transforms from an epithelial to a mesenchymal morphology, and participates in the formation of the valves and membranous septa. The VSD observed in jmj-/- embryo may result from an endocardial cushion (EC) defect, which leads to a lack of fusion of the EC to the basal part of the septum. The observed DORV may result from abnormal EC formation. Therefore, our jmjen mice provide a powerful tool to examine the unexpected endothelial-specific roles of jmj in cardiac development.

175. The contribution of second heart field cells to the jaw muscle reveals the multi-potential nature of the cardio-craniofacial mesoderm

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Recent studies demonstrated that common progenitor populations of mesoderm cells in the head contribute to both cardiac and skeletal muscle lineages, suggesting that developmental multipotentiality is more pervasive than previously expected. In order to understand the cellular and molecular underpinnings of this phenomenon, we utilized several fate-mapping techniques along with gene expression analyses in chick embryos, to systematically track both cardiac and skeletal muscle precursors. We demonstrate that the cardiac progenitor population, known as the second heart field (SHF), lies between the cranial paraxial mesoderm (CPM) and the lateral splanchnic mesoderm (first heart field or FHF) at cardiac crescent stages. During gastrulation, these SHF cells are segregated with the lateral mesoderm, and marked by the expression of Isl1, Nkx2.5, Tbx20 and Fgf10. We further provide cellular and molecular insights into the regionalization of the pharyngeal mesoderm within the branchial arches by both SHF and CPM cells: CPM cells fill the proximal region of the myogenic core, whereas SHF cells populate the distal portion of this core. Moreover, our findings reveal distinct developmental programs for CPM-derived and SHF-derived branchiomeric muscles. While both muscle types express Myf5 and MyoD, CPM-derived muscles (e.g., Mandibular Adductor) express Pax7, SHF-derived muscles (e.g., Intermandibular) express Isl1 along with a delayed MHC expression. In addition, ectopic activation of the Wnt/B-catenin pathway resulted in a cardiac looping phenotype along with inhibition of Nkx2.5 and Isl1 expression in the SHF, suggesting a role for this signaling pathway in the regulation of the second heart field. These experiments provide the first insights into the processes underlying the development of the cardio-craniofacial mesoderm during embryogenesis.

176. Tamoxifen-inducible expression of Cre-recombinase in the early definitive endoderm in mice
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In order to employ conditional mutagenesis to interrogate gene function and circumvent embryonic lethality during mouse development, temporospatially restricted Cre drivers are crucial. We have generated a Cre-expressing mouse that takes advantage of the temporally inducible nature of mER;Cre;mER (Reth, 1996). This is a fusion protein whose nuclear translocation is dependent on occupation of the mutant ER by artificial ligand (tamoxifen). We targeted mER;Cre;mER with an IRES to the Foxa2 locus to generate the Foxa2MCM allele. We have previously used this strategy to obtain endodermal expression of the rtTA (Frank et.al, 2007). Foxa2 is expressed in multiple locations during early embryogenesis including the visceral endoderm, definitive endoderm, ventral neural tube and notochord. To examine Cre activity, we crossed Foxa2MCM with Rosa26R reporter mice and embryos were assayed for β-galactosidase activity. Activity of Foxa2MCM recapitulates endogenous Foxa2 expression and no reporter gene expression was detected in untreated embryos. Dose-dependent recombination was detected. We also found that the method of tamoxifen administration affected Cre activity by comparing efficiency and location of Cre activity after administration of tamoxifen by oral gavage versus intraperitoneal injection. By varying the timing of tamoxifen administration, we obtained Cre activity in different Foxa2 expression domains. We are also employing this system to investigate the lineage of Foxa2 expressing cells. This system will be useful for controlling the activity of floxed alleles at multiple stages of mouse embryogenesis and fetal development.

177. Abnormal myocardial secretory function and failed EMT in the outflow tract contribute to Persistent Truncus Arteriosus in Fgf8 conditional mutant mice
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We are investigating the molecular and cellular events regulated by Fgf8 and required for septation of the truncus arteriosus. Conditional ablation of Fgf8 in mesoderm and endoderm with Isl1Cre results in 100% incidence of persistent truncus arteriosus. The present studies reveal the multifactorial basis of this phenotype. At e10.5, both the proximal and distal outflow tract (OFT) cushions are hypocellular and exhibit a reduced quantity of cardiac jelly. Lineage analyses indicate that the proximal cushions are populated by endothelial-derived cells and the distal cushions by neural crest at this stage. We examined the ability of OFT cushion endothelial cells to undergo EMT using an OFT explant system. Explant cultures derived from Fgf8;IslCre mutant OFTs (N=12) fail to execute endothelial EMT, while EMT in the atrioventricular cushion was identical to controls. Although the total amount of cardiac jelly is decreased in mutants, there is increased deposition of periostin in the matrix. Furthermore, Wnt11 expression is decreased in the mutant OFT myocardium. All of these findings point to an abnormal secretory program in the OFT myocardium, and suggest that the neural crest and endothelial defects are non cell-autonomous. This hypothesis is consistent with our Fgf receptor ablation studies that identify anterior heart field mesoderm as a crucial target of Fgf8 signaling to mediate OFT septation. OFT septation occurs in the absence of functional FgfR1 and/or 2 in neural crest and endothelium. We are employing the explant co-culture system to determine if the endothelial EMT defect can be rescued by matrix or secreted signaling factors from control explants. We are also investigating the polarization, secretory apparatus, and cellular structure of OFT myocardium and quantitatively and qualitatively assessing the composition of the OFT cardiac jelly to identify signaling pathways disrupted by loss of Fgf8.

178. Sonic hedgehog and vascular progenitor cells

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The walls of muscular arteries are composed of three distinct layers, the intima, media, and adventitia. Whole mount staining in Ptch1-lacZ knock-in mice reveals active hedgehog signaling within the adventitia of artery walls beginning at midgestation. When Shh distribution was assessed by immunofluorescence, a distinct pattern of staining was found at the border between the aortic media and adventitia. Sca1+ progenitor cells (AdvSca1 cells) were found co-localized with Shh and Ptch1-lacZ-positive cells in the adventitia of developing arteries. Analysis of Shh-/- mice indicates that the number of AdvSca1 cells is reduced in the absence of Shh. Wild-type AdvSca1 cells do not express SMC markers in vivo, yet they differentiate to SMCs in culture. Despite the absence of SMC marker expression in vivo, a subset of AdvSca1 cells express serum response factor and myocardin, factors thought to be sufficient for expression of many SMC marker genes. Consistent with the idea that SMC marker genes are actively silenced, AdvSca1 cells express multiple repressors of SMC marker gene transcription, including the Shh target gene Msx2. Our results suggest that Shh signaling in the adventitia plays a role in establishing and/or maintaining a population of progenitor cells which could participate in repair and remodeling of the artery wall.

179. Eomesodermin cooperates with Smad2 to activate a subset of mesodermal target genes in Xenopus embryos

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HYPOTHESIS: Eomesodermin (Eomes) cooperates with activated Smad2, through physical association of Eomes with Smad2, to activate its mesodermal target genes. BACKGROUND: Eomes is an early mesodermal gene first identified in Xenopus, and is both necessary and sufficient for mesoderm differentiation in Xenopus, mouse and zebrafish. Eomes encodes a T-box transcription factor of the T-brain1 subfamily of DNA-binding T-box genes. Eomes has recently also been shown to be important in endoderm formation in zebrafish, CD8 T-cell differentiation in mice, and cardiac development in Xenopus. RESULTS: 1. Eomes requires TGF-beta signaling (dominant-negative activin receptor) to activate mesodermal (Xbra, Xwnt8) and mesendodermal (Mix.1) genes. 2. Recombinant human activin protein works well in Xenopus animal pole explant (animal cap) assays. 3. A dominant negative Smad2 isoform (P445H) blocks activin signaling (caps). 4. Eomes requires TGF-beta receptor-regulated phosphorylation of Smad2 to activate genes restricted to Spemann's Organizer (goosecoid, chordin, Xnr1, and Xnr2), and the myogenic gene Myf5 (partial requirement). 5. Direct targets of activation by Eomes include Xwnt8 (ventral mesoderm), Xnrs 1, 2 and 5 (Organizer), and Mix.1 (mesendoderm). 6. Eomes, Xbra, gsc and Sox17-beta are variably de-repressed by inhibition of protein synthesis (cycloheximide), precluding determination of whether these genes represent direct Eomes targets. CONCLUSIONS: Eomes operates in early embryogenesis via both Smad2-dependent and -independent mechanisms. Our results have implications for differentiation of mesoderm, endoderm, and cardiac development.

180. A Novel TBX5 Mutation Leads To Enhanced DNA-binding In A Family With A-typical Holt-Oram Syndrome And Paroxysmal Atrial Fibrillation

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Background: Holt-Oram syndrome (HOS) is a heart-hand syndrome clinically characterized by upper limb- and cardiac malformations. Mutations in TBX5, a member of the T-box transcription factor family,

were shown to underlie this syndrome. Here we describe a HOS family of 13 members with paroxysmal atrial fibrillation and demonstrate that a novel TBX5 mutation co-segregates with affected family members, displays enhanced DNA binding affinity, and significantly augmented Nppa and Cx40 promoter activity. Methods and results: Affected family members are characterized by autosomal dominant radial luxation, carpal fusion, scapular dysplasia and variable heart defects (ASD/VSD) without thumb malformations. The majority of the affected family members, including those without (haemodynamically significant) structural heart defects, also developed paroxysmal atrial fibrillation. Sequencing of TBX5 revealed a novel mutation (p.Gly125Arg), in all affected family members, located in the conserved T-box binding element and absent from over 200 controls. Trafficking experiments showed that the mutant TBX5 protein resides in the nucleus. Subsequent EMSA studies demonstrated that the DNA binding affinity of the mutant TBX5 is higher than wildtype TBX5. Moreover, the G125R TBX5 mutant shows a significantly enhanced activation of both the Nppa promoter and the Cx40 promoter in comparison to wildtype TBX5. Conclusions: We describe a novel TBX5 mutation in a family with features of HOS. Contrary to previously published mutations, the underlying p.Gly125Arg TBX5 mutant shows a higher DNA-binding activity, which suggests that the effect of this mutation is through a gain of function mechanism. Interestingly, the majority of the affected family members display paroxysmal atrial fibrillation. Taken together with the fact that the mutant TBX5 augments the activity of TBX3, HCN4, CX40 and CX43, this could hint at a possible role of TBX5 in the development of (paroxysmal) atrial fibrillation in this family.

181. Targeted deletion of the zebrafish obscurin RhoGEF domain impairs heart, skeletal muscle, and brain development

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Obscurin is a giant structural and signaling protein demonstrated to be required for the assembly and structural integrity of striated myofibrils. Depletion of obscurin in developing zebrafish embryos resulted in misalignment of adjacent myofibrils and disorganization of the surrounding sarcoplasmic reticulum. Previous work has examined the physical interactions between obscurin and other cytoskeletal elements but its role in cell signaling, including the functions of its RhoGTPase Exchange Factor (RhoGEF) domain have not been characterized. In this study, we sought to determine if the functions of the obscurin RhoGEF domain were required during vertebrate development. To ablate obscurin RhoGEF activity, we used two independent morpholino antisense oligonucleotides to target the exon encoding for the active site of the obscurin RhoGEF domain. Injection of either morpholino alone resulted in a phenotype very similar to that previously noted using morpholinos targeting the translation initiation site. By comparison, co-injection of the two morpholinos targeting the RhoGEF exon resulted in a milder skeletal muscle phenotype while maintaining the more severe defects of cardiac and central nervous system development. Embryos injected with both morpholinos demonstrated increased cell death within the developing brain and neural tube and mislocalization of the small GTPase RhoA as evidenced by acridine orange staining and immunostaining respectively. RT-PCR analysis demonstrated efficient in-frame deletion of the targeted exon, and immunostaining demonstrated intact localization of obscurin carboxy terminal epitopes suggesting that the exon deletion had left the carboxy terminal structural domains intact. mRNA injection of a mini-construct encoding for the obscurin RhoGEF and carboxy terminal localization domains rescued the observed phenotype. These results demonstrate that signaling by the obscurin RhoGEF domain is required for normal brain and striated muscle development and that its effects may be mediated in part through the localization and activation of small GTPases like RhoA at specific intracellular sites.

182. Temporal attenuation of ALK4 signaling in Xenopus reveals direct and indirect effects of left-right axis perturbation on heart development

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Body situs defects in heterotaxy almost always are accompanied by congenital heart defects (CHDs), suggesting critical roles for laterality genes in heart development. However, because laterality genes typically are expressed at multiple times and in multiple locations within the embryo—including the heart itself—it is unclear whether the CHDs arise as primary or secondary defects of aberrant left-right axis determination. To distinguish between these possibilities, we re-examined the role that the type I TGF-beta receptor, activin-like kinase 4 (ALK4), plays in L-R development. In Xenopus, ALK4 is expressed in both cardiac and non-cardiac tissues. Ectopic expression of RNA encoding constitutively active or dominant-negative ALK4 (but not other closely related ALKs) causes heterotaxy and a range of CHDs, including defective chamber formation, abnormal ventricular trabeculation, decreased septal tissue formation, and maldeveloped outflow tract. To achieve temporally targeted attenuation of ALK4 signaling, a pharmacological ALK4/5/7 inhibitor, SB 431542, was added to embryos at defined developmental stages. Embryos exposed to SB 431542 during pre-gastrulation stages (st. 1-9) developed normal body situs. However, exposure during stages encompassing either early neurulation (st. 13-18) or heart field formation (st. 20-25) caused significant heterotaxy. Hearts from these embryos showed structural defects; however, unlike CHDs resulting from ectopic RNA expression, the CHDs in SB 431542 treated embryos were present only in the outflow tract (st. 13-18 treatment) and/or atria (st. 13-18 and 20-25 treatments). Later drug exposure (st. 20-25) additionally caused a small percentage of AV cushion defects. Together, these results demonstrate that perturbation of ALK4 signaling either before or during early stages of heart development can cause both L-R situs and cardiac defects. In turn, the types of CHDs present differ according to the temporal window in which ALK4 signaling activity is perturbed, suggesting that defective ALK4 signaling can both directly and indirectly elicit CHDs in heterotaxy embryos.

183. Cardiomyocyte specification and terminal differentiation are compromised by left-right axis perturbation in Xenopus

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The morbidity and mortality of heterotaxy are frequently attributed to complex congenital heart defects (CHDs), reflecting the extreme susceptibility of the developing heart to L-R axis perturbations. Although much progress has been made in defining the genetic and cellular processes that establish the L-R body axis, very little is known about how L-R axial pathways influence cells during cardiac morphogenesis. To address this, we are investigating three functionally and temporally unrelated L-R axial signaling pathways in Xenopus to determine which processes of cardiogenesis are affected, and whether the different pathways affect them in the same way. Embryos treated with a serotonin receptor inhibitor (GR 113808), a H+/K+-ATPase inhibitor (SCH 28080), or an ALK4/5/7 inhibitor (SB 431542), develop heterotaxy in addition to structural CHDs. Sibling treated embryos from these groups were processed by in situ hybridization just prior to heart tube formation (st. 28/29) to analyze Nkx 2.5 and myosin light chain-2 (mlc-2) expression. GR 113808 or SCH 28080 treated embryos exhibited reduced Nkx 2.5 staining; however, the regions of reduced staining differed between the groups. By contrast to its normal, bilaterally symmetric pattern, mlc-2 was shifted to left-dominant in GR 113808 treated embryos, and to right-dominant in SCH 28080 treated embryos. SB 431542 treated embryos had right-dominant Nkx 2.5 staining; however, Nkx 2.5 staining was affected minimally, if at all, in embryos with ectopically activated ALK4. Right-dominant mlc-2 also was present in SB 431542 treated embryos; however, ectopic ALK4 signaling caused left-dominant mlc-2. Together, these results demonstrate that expression

of genes that mark cardiomyocyte specification and differentiation is uniquely compromised by perturbation of L-R axis determination. The convergence of three functionally and temporally unrelated L-R signaling pathways on these processes moreover suggests that they are crucial targets in the development of CHDs associated with heterotaxy.

184. The trabeculated right ventricular free wall in the chicken heart forms by ventricularization of the myocardium initially forming the outflow tract

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Recent molecular lineage analyses in mouse have demonstrated that the right ventricle is recruited from anterior mesoderm in later stages of cardiac development. This is in contrast to current views of development in the chicken heart, which suggest that the initial heart tube contains a subset of right ventricular precursors. We investigated the fate of the OFT myocardium using immunofluorescent staining of the myocardium, and lineage tracer, as well as cell death experiments. These analyses showed that the outflow tract is initially myocardial in its entirety, increasing in length up to HH24. The OFT myocardium, subsequently, shortens as a result of ventricularization, contributing to the trabeculated free wall, as well as the infundibulum, of the right ventricle. During this shortening, the overall length of the OFT is maintained due to the formation of a non-myocardial portion between the distal myocardial border and the pericardial reflections. Cell death and transdifferentiation were found to play a more limited contribution to the initial shortening than is generally appreciated, if they play any part at all. Cell death, nonetheless, plays an important role in the disappearance of the myocardial collar that continues to invest the aorta and pulmonary trunk around HH30, and in the separation of the intrapericardial arterial vessels. Taken together, we show, as opposed to some current beliefs, the development of the arterial pole is similar in mammals and birds.

185. CMF1-Rb interaction in myogenesis

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CMF1 (avian) and LEK1 (mouse) are proteins expressed in embryonic striated muscle with onset of expression preceding that of contractile proteins. Our laboratory and others' have shown that disruption of CMF1 or LEK1 in myoblasts disrupts muscle-specific protein expression. Preliminary studies indicate that an Rb-binding domain within the C-terminus of CMF1 and of LEK1 is functional, and that interaction with Rb may mediate the role of these proteins in myocyte differentiation. Here we examine in detail the Rb-binding function of CMF1, using a CMF1 Rb-binding domain-deleted stable myogenic cell line. These cells proliferate normally and synchronize in response to low serum medium, as do wild-type myocytes. However, they exhibit a markedly impaired capacity to differentiate. Specifically, they express less contractile protein than wild type cells, and do not fuse into myotubes. MyoD and Myf5 mRNA levels are similar to wild-type by quantitative realtime PCR, but myogenin and contractile protein mRNA levels are significantly attenuated. These data suggest that CMF1 coordinates with Rb in regulating the transition from proliferation to terminal differentiation in embryonic muscle. We are currently testing this hypothesis for LEK1 in a murine ES cells model of cardiomyocyte differentiation. We show that LEK1 is highly expressed in myosin positive ES cells. A time-course of expression shows that LEK1 mRNA increases as ES cells differentiate into cardiomyocytes. Further, the timing of this expression is accelerated when noggin is added to enhance differentiation into the cardiomyocyte lineage. We are now turning to the study of ES cells with targeted disruption of LEK1 and of the LEK1 binding domain.

186. Determination of the Cardioteratogenic Window for Low Dose Trichloroethylene Exposure to Chick Embryos

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Trichloroethylene (TCE) is the most frequently reported groundwater contaminant. Several studies associate gestational TCE exposure with congenital heart defects, including valvuloseptal malformations; however, this conclusion is highly controversial and the basis for TCE's cardiac teratogenicity is not well understood. We determined that chick embryos exposed in ovo to 8 ppb TCE during early valvuloseptal morphogenesis (Hamburger and Hamilton (HH) stages 13 - 20) have reduced cardiac output at HH24, hyperplastic cushions, and increased mortality by HH30, whereas exposure during the specification period (HH3-17) was ineffectual at disrupting heart development. To identify the critical window of cushion sensitivity, we exposed embryos to TCE at a single developmental stage (HH 13, 15, 17, 20, or 24) and assessed survival at HH30. TCE exposure at HH 13 or 15 had little effect upon survival to HH30, whereas survival was significantly reduced following treatment at HH 17, 20, or 24. Sensitivity to TCE was greatest at HH17, and these embryos exhibited a U-shaped dose-response curve with greatest mortality at 8 ppb, a dose just above the EPA safe limit for humans. Current studies evaluate embryos exposed to this dose (8 ppb, HH17) with respect to valvuloseptal and calcium regulatory gene expression, and cardiac function at hatch via echocardiography. Our initial echocardiography evidence, which was confirmed by anatomical analyses, indicates a high incidence (7 of 7 chicks) of ventricular septal defects in hatched chicks following HH17 TCE exposure. Our data reinforce that TCE is a cardiac teratogen for chick, and point to events during cushion invasion and cardiocyte expansion as potential targets of this common groundwater toxicant. (Supported by NIH award #ES11738.)

187. Genome-wide identification of Nkx2.5 target genes using Chromatin immunoprecipitation and DNA microarrays

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Chromatin immunoprecipitation (ChIP) is a powerful technique that enables the mapping of transcription factor binding in vivo. Cells or tissue are crosslinked in vivo capturing the protein-DNA-interactions, the DNA is fragmented and specific protein-DNA complexes are immunoprecipitated using a transcription factor specific antibody. By coupling ChIP with DNA microarrays (ChIP-on-chip) it is possible to map the genome-wide binding of specific transcription factors in vivo, and to identify genes that may be directly regulated by that transcription factor. Here we demonstrate the use of this technique to study the transcriptional regulation of cardiogenesis in the developing mouse embryo. Furthermore, we have used ChIP-on-chip to map the in vivo binding sites of the transcription factor Nkx2-5 at the key developmental stages of E9.5 and E11.5 using a genomic mouse promoter array. The promoter array used in this study contained 3kb upstream of approximately 13000 genes. From analysis of these experiments we have identified 42 and 48 promoter regions bound by Nkx2-5 at E9.5 and E11.5 respectively. We are now confirming the binding of Nkx2-5 to several targets identified in the location analysis by ChIP using quantitative PCR. To date 10 promoter regions identified as being bound by Nkx2-5 have been tested, and 9 out of 10 have shown enrichment in Nkx2-5 immunoprecipitated DNA compared to control, clearly validating this approach. We are currently using bioinformatics methods to examine the identified promoter fragments for Nkx2-5 binding and for other binding sites of transcription factors that may be over represented.

188. DiGeorge Syndrome-Like Outflow Tract Phenotype Induced by Maternal Administration with Retinoic Acid

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Deficiency and excess of retinoic acid (RA), the biologically active form of vitamin A, have profound effects on the embryonic development of cardiovascular systems. Mouse embryos treated with RA at embryonic day 6.5 (E6.5) induce visceroatrial heterotaxy syndrome characterized by abnormalities in laterality, looping, and formation of atrioventricular cushion development, which secondarily influences ventricular chamber and outflow tract development (Yasui, Morishima et al., 1998). On the other hand, mouse embryos treated with RA at E8.5 induce transposition of the great arteries by impairing the initial formation of the conus cushions leading to a loss of spirality in the cardiac outflow tract (Yasui, Morishima et al., 1995). Here we show that RA treatment at E9.5 affected the normal cardiac outflow tract formation and resulted in DiGeorge syndrome-like phenotype. RA (70mg/kg) was intraperitoneally injected into pregnant ICR mice at E9.5. The cardiovascular abnormalities in the RA treatment at E9.5 included persistent truncus arteriosus, tetralogy of Fallot, double outlet right ventricle, aorto-pulmonary window, perimembranous ventricular septal defect, and aortic arch abnormalities such as interrupted aortic arch, coarctation of aorta, right aortic arch, and aberrant subclavian artery. Extracardiac anomalies in RA treatment at E9.5 included craniofacial anomalies such as cleft palate, low set ear, short neck, and anomaly and hypoplasia of thymus, malrotation of the intestine, and short tail. We were able to establish the DiGeorge syndrome-like outflow tract phenotype in mice treated with RA at E9.5. To understand what kind of genes are involved in the abnormal formation of the outflow tract in this model, we are now investigating gene expression profiles especially of the outflow tract and the secondary heart filed by the use of DNA microarray.

189. Sca-1 cardiac progenitor cells do not differentiate into cardiac myocytes

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Recent studies have indicated that Sca-1+ cardiac progenitor cells may differentiate into cardiac myocytes in culture and in an ischemia-reperfusion model. In this study we determine survival, differentiation status and functional coupling of Sca-1+ cardiac progenitor cells grafted into the infarct border zone of recipient hearts. Hearts were removed from double transgenic α-MHC-nLac (cardiac-myocyte specific nuclear lac-z) x β-actin EGFP (ubiquitous EGFP) adult mice. A myocyte-depleted population was generated by enzymatic digestion and was then sorted for EGFP and Sca-1 positivity and lineage-marker negativity. Syngeneic mice underwent coronary artery ligation and were immediately engrafted with 100,000 sorted cells in the infarct border zone. Mice were sacrificed between 11 and 21 days post-surgery. As demonstrated by EGFP epiflourescence, 10 out of 11 mice harbored intra-myocardial grafts. The MHC-nLac reporter was not activated in any of the engrafted cells, indicating that they did not differentiate into cardiac myocytes. Two-photon laser scanning microscopy of [Ca2+]i sensitive dye-loaded hearts indicated that there were no intracellular calcium transients in grafted cells during either point or field stimulation. These data indicate that within this system Sca-1+ cardiac progenitor cells may be grafted successfully into the border zone of recipient mice. These cells do not differentiate into cardiac myocytes, and do not functionally couple with the host myocardium.

190. Imaging Cellular Calcium In The Embryonic Murine Heart Using A Genetically Encoded Calcium Indicator

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Genetically encoded Ca2+ sensing proteins are a novel tool to study complex cellular interactions within an organ. Recently we described conditional cardiac expression of a genetically encoded Ca2+ indicator, GCaMP2, which is stable at 37ºC, has brightness similar to eGFP, and a large dynamic range (Tallini Y et al PNAS 2006). We have used conditional cardiac GCaMP2 (ccGC) mice to examine early conduction events in the embryonic heart before and after formation of the AV node. At 10.5 days post coitus (dpc) Ca2+ transients propagated rapidly through the atria and slowed distinctly as they traveled along the surface of the atrioventricular canal (AVC); ventricular activation occurred rapidly following propagation through the canal. Premature ventricular activations resulted in retrograde conduction through the canal with an equivalent slowed conduction rate. Mice expressing GCaMP2 under control of the connexin40 locus (Cx40BAC-GCaMP2 transgenic mice) demonstrated a lack of signaling from within the AVC and an abrupt loss of expression at the entrance to the canal. The slowing of the transmission through the AVC at this early stage acts as an effective AV delay prior to a morphologically distinct AV node. However, at 13.5 dpc the wave of cellular activation terminates at the junction of the atria and ventricles and emerges at the ventricular apex, resulting in a markedly shortened AV delay. Moreover, retrograde transmission either associated with premature ventricular activation or ventricular pacing did not result in retrograde transmission to the atria. These studies demonstrate the importance of Cx40 expression for early embryonic conduction and the critical role of the AVC in establishing an AV delay prior to complete organogenesis.

191. Transcription Factor Ap2d Associates with Ash2l and ALR, a Histone Methyltransferase, to Activate Transcription During Development

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The Ap2 family of transcription factors has been shown to be associated with CHD. Ap2a is known for its role in the cardiac neural crest, and its deficiency causes severe developmental defects such as persistent truncus arteriosus. TFAP2D was identified through a homology search using TFAP2B, the disease-causing gene for Char Syndrome characterized by patent ductus arteriosus. Tcfap2d, which encodes Ap2d, is expressed in the myocardium during a short temporal window but is absent in the neural crest where other Tcfap2 genes are expressed. Its expression pattern indicates that Ap2d may have a novel role in cardiac development that is distinct from other Ap2 family members. To characterize Ap2d, we performed a yeast two-hybrid screen that identified Ash2l, a Trithorax superfamily member involved in chromatin remodeling, as a potential co-activator of Ap2d involved in regulating gene targets throughout development. By co-IP, we showed that Ap2d interacts with full-length Ash2l in 293T cells and that this interaction is specific for Ap2d, as complexes containing Ap2a, -b, -g, and -e and Ash2l were not observed. To determine the role of Ash2l as a co-activator, we showed that Ash2l has a dose-dependent effect on Ap2d-mediated gene regulation. Furthermore, endogenous co-IP experiments revealed that Ap2d associates with both Ash2l and the histone methyltransferase ALR in K562 cells and that this complex is able to methylate recombinant histone H3 in vitro. In situ hybridizations show that Ash2l expression overlaps with that of Tcfap2d during embryogenesis. In summary, we show that Ap2d is distinct from other Ap2 proteins in that it interacts through its poorly conserved transactivation domain with Trithorax superfamily members, Ash2l and ALR, which are involved in activating transcription through H3-K4 methylation.

192. Inter-atrial communication between right juxtaposed atrial appendages

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Juxtaposition of the atrial appendages is a relatively rare anomaly where both atrial appendages lie to the right or the left of the great arteries. Right juxtaposition of the left atrial appendage is less common. We present an unusual case of right juxtaposition of the left atrial appendage with an anomalous interatrial communication between the closely approximated appendages. The anomaly was identified at post mortem in a 36 week gestation fetus in which severe hydrocephalus had been identified prenatally. Chromosome results were for a normal male, 46, XY. The communication between the atrial appendages measured 0.4 x 0.2 cm viewed from the right side and was located at the base of the right atrial appendage. From the left, the opening was slightly distal to that appendage's connection with the body of the left atrium and was divided by a small band of tissue creating two openings, measuring 0.3 x 0.3 cm and 0.2 x 0.1 cm. Other than this intercommunication, the interiors of the atrial appendages were completely separate. Externally, the left atrial appendage was joined to the right except for the distal 0.15 cm of the left atrial appendage tip which was free. Other cardiac findings included a moderately hypoplastic left heart, which did not form part of the cardiac apex. Both atrioventricular and ventriculoarterial connections were concordant, the oval foramen was patent and no interventricular defects were noted. To our knowledge, this type of interatrial communication has not been previously described. Presumably, it was due to conjoining of the atrial appendages during development as a result of their close juxtaposition.

193. Hand2 Is Required for Second Heart Field Development During Cardiogenesis

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The basic helix loop helix (bHLH) family of transcription factors plays important roles in the determination and differentiation of multiple organs during development. Hand2, also known as dHAND, of the HAND subclass of the bHLH family, is expressed throughout the heart with dominant expression in the right ventricular segment derived from the second heart field. Hand2-null embryos die around E10.5 due to heart failure and display severe hypoplasia of the right ventricle and poor trabeculation in the left ventricle. However, the early embryonic lethality of the Hand2-null embryos has precluded the study of its function in heart development at later stages. To determine the function of Hand2 in specific domains and at later stages of cardiogenesis, we generated mice with loxp sites surrounding the Hand2 gene. Hand2-loxp mice were mated with three different cardiac-specific Cre lines—Tbx1Cre and Mef2cCre, which excise floxed genes in the second heart field, and Nkx2.5Cre, which is active in both right and left ventricles. The resulting tissue-specific mutants are all embryonic lethal, but survive longer than Hand2-null mutants. We observed that tissue-specific Hand2 mutants had various degrees of right ventricular and outflow tract defects. Tbx1Cre;Hand2 mutants died around E15.5 and displayed conotruncal defects with a small right ventricle. Mef2cCre;Hand2 mutants died around E13.5 with a muscular defect of both ventricles. Nkx2.5Cre;Hand2 mutants died around E12.5 and displayed a more severe ventricular muscle defect than Mef2cCre;Hand2 mutants. Analyses of gene expression alterations and relationship of mutants to defects in second heart field development will be presented.

194. Loss of Tbx1 in pharyngeal ectoderm recapitulates 22q11.2 deletion syndrome defects
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22q11DS is a chromosomal disorder characterized by congenital defects of the heart, great arteries, craniofacies, thymus and parathyroids. TBX1 is the major gene for 22q11DS. In the mouse, Tbx1 is widely expressed in the pharyngeal apparatus (PA), an embryonic structure that contributes to most structures affected in 22q11DS. We are using tissue-specific gene deletion to pinpoint where Tbx1 is required for development of the PA derivatives. Our previous studies suggest that pharyngeal (ph) epithelia (ectoderm and endoderm) may be critical domains. Here, we have focused on the requirement for ectodermal Tbx1. We have deleted Tbx1 using three Cre-drivers that are differentially expressed in ph. epithelium and neural crest (NCC); Fgf15TgCre is expressed in ph. ectoderm and endoderm, Ap2αCre is expressed in ph. ectoderm and NCC, Wnt1Cre is expressed in NCC. Wnt1Cre deletion of Tbx1 caused no abnormality in conditional heterozygous and null mutants, consistent with reported lack of Tbx1 expression in NCC. Thus, we deduce that abnormalities that may be found in Ap2αCre-deleted conditional mutants would be due to loss of ectodermal Tbx1. Surprisingly, Ap2αCre conditional mutants had a spectrum of 22q11DS-like malformations, namely, aortic arch defects, thymic hypoplasia and cleft palate. Thymic defects were unexpected and when considered with other published data, suggest that all three germ layers contribute to thymic development. Ap2αCre-deleted conditional heterozygotes recapitulated the 4th arch artery haploinsufficiency phenotype, demonstrating that Tbx1 expression in ph. ectoderm is both necessary and sufficient for 4th arch artery development. Therefore, although Tbx1 is expressed early and transiently in ph. ectoderm, it plays a critical role in PA development.

195. Non-viral delivery of frataxin to mitochondria for Friedreich's Ataxia
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Friedreich's Ataxia (FA) is the most common cause of inherited human ataxia. It is caused by a deficiency of the protein, Frataxin, arising from a triplet GAA expansion in intron-1 of the FRDA gene. It results in iron accumulation and possible oxidant stress in mitochondria leading to progressive cardiomyopathy and ataxia. Therapies have not been effective and patients die from hypertrophic cardiomyopathy at a young age. We have used novel protein transduction domains (PTD), notably TAT, to deliver Frataxin to mitochondria both for cells in culture, and in vivo. PTD's are short, positively charged peptides that are highly efficient at delivering protein cargos across cell membranes. This transduction into mitochondria does not require energy or receptors, and is dependent on the concentration gradient of the TAT-fusion protein. To date, we have developed a TAT-Frataxin fusion protein and have shown it will target mitochondria of human Frataxin deficient fibroblasts in culture, and in mice deficient in cardiac Frataxin. The Frataxin remains in mitochondria in excess of 21 days (in culture), and does not induce an inflammatory response in mice when injected chronically in vivo over 2 months. TAT- Frataxin treated fibroblasts from FA patients also showed reduced caspase-3 activation and cell death compared to controls when treated with iron as an oxidant stress. These data show a functional protein can be delivered to mitochondria via PTD and support the hypothesis that TAT-Frataxin can be developed as a novel therapy for the treatment of Friedreich's Ataxia using protein transduction domains. Further experiments are in progress to rescue the phenotype of the conditional FA knockout mouse.

196. Wnt3a and Wnt8.2 Regulate the Formation of Cardiac Neural Crest Cells in Zebrafish
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The cardiac neural crest is a subregion of the dorsal neural tube composed of cells that migrate to the heart where they contribute to distinct aspects of cardiogenesis. However, the formation of this group of

cells is not clear . Here we demonstrate that this particular group of CNC cells is induced by canonical Wnt signaling. Wnt3a and Wnt8.2, in the formation of the cardiac neural crest in zebrafish through regulation of crip2 and sema3d, two cardiac neural crest markers. Whereas expression of sema3d demarcates cardiac neural crest cells in rhombomeres 3 – 5, crip2-expressing cells originate in the dorsal part of rhombomere 6 and then migrate to the primary heart field, where they contribute to the formation of cardiomyocytes. Similarly, wnt3a and wnt8.2 exhibit spatially and temporally restricted expression patterns in the hindbrain region. Further, genetic evidence indicated that both wnt3a and wnt8.2 are involved in determination of the cardiac neural crest cell fate, with wnt3a being required for the induction of both crip2 and sema3d, while wnt8.2 functioned downstream of wnt3a and was only required for the induction of crip2. Together, these data reveal a role for canonical Wnts in cardiac neural crest induction by regulating the expression pattern of specific downstream target genes.

197. RDC1/CXCR7 regulates proliferation of semilunar valve mesenchymal cells in mice

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RDC1/CXCR7 is a seven-transmembrane G protein-coupled receptor (GPCR) with conserved motifs characteristic of chemokine receptors. It is expressed in various tissues including brain, heart, kidney, spleen and thymus. To test this hypothesis, we generated RDC1-flox mice that delete the entire coding region of RDC1 after recombination. Ubiquitous disruption of RDC1 resulted in perinatal lethality with complete penetrance. We found that newborn and embryonic day 18.5 (E18.5) RDC1-null embryos exhibited aortic and pulmonary valve stenosis due to thickening of the semilunar valves and the mutants also had occasional ventricular septal defects; the tricuspid and mitral valves were normal. Migration of neural crest cells to the heart through pharyngeal arches 3, 4 and 6 was also normal in RDC1-null embryos. The proliferation rate of semilunar valve mesenchymal cells was increased in mutants from E14 onward but the cell death rate remained unchanged. We found that the level of phospho-Smad1/5/8 was increased in RDC1 mutant valves, consistent with evidence for Bmp signaling in regulating valve proliferation. Endothelial/endocardial-specific deletion of RDC1 using the Tie2-Cre transgene resulted in semilunar valve stenosis, albeit a milder form, isolating RDC1 function in the endocardium and its mesenchymal derivatives as essential for regulating valve proliferation and remodeling. These findings suggest that the GPCR chemokine receptor RDC1 plays a central role in semilunar valve development, possibly by regulating Bmp signaling, and may contribute to diseases such as aortic and pulmonary valve stenosis.

198. Impaired Cx43 Expression and Cardiomyocyte Differentiation in BMPR2 Mutant Cells and Embryos

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A hypomorphic allele of the common receptor for Bone Morphogenetic Proteins (BMP), BmpRIIΔE2, results in outflow tract (Délot et al., 2003) and atrioventricular canal (see poster by Kruithof et al.) developmental defects in the mouse embryo. However, another phenomenon may be the cause of the early (starting at E11.5) death of these embryos. Searching for downstream effectors of BMP in cardiac differentiation, we found that Cx43 was upregulated in homozygous BmpRIIΔE2 embryonic hearts. We used the ability of P19 embryo carcinoma cells to mimic mesoderm induction upon aggregation and differentiation into cardiomyocytes upon DMSO treatment to assess whether mutant Bmpr2 might modify differentiation potential. Stable transformants of P19 cells carrying either the wild type or ΔE2 receptor were established. No difference in the number of MF-20-positive cells at day 6 between P19-WT and P19-ΔE2 showed that BMP signaling is not necessary for early cardiomyocyte marker induction. However, by day 14 impaired BMP signaling abolished expression of cardiomyocyte differentiation

markers myosin heavy chain, Gata-4 and Tbx5, and beating colonies were almost totally absent in mutant cells. Aggregation of naive P19 cells was accompanied by a dramatic decrease of Cx43 expression, and this was amplified by over-expression of BmprR2-WT. But at late stages of differentiation, Cx43 protein expression was up-regulated in P19-ΔE2 cells (as it was in vivo). Co-immunostaining for Cx43 and MF-20 showed distinct patterns of expression, suggesting that Cx43 might be also involved in other events independent of cardiomyocyte differentiation or that its effect is not cell autonomous. This novel association of BMP impairment and dysregulation of Connexin expression, both in vitro and in vivo, suggests a developmental link between BMP and Cx43 that could explain the outflow tract anomalies and/or the premature death of Bmpr2ΔE2 homozygotes.

199. A Novel Role of BRG1 in the Regulation of SRF/MRTFA-Dependent Smooth Muscle-Specific Gene Expression

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Serum Response Factor (SRF) is a key regulator of smooth muscle differentiation, proliferation and migration. Myocardin Related Transcription Factor A (MRTFA) is a co-activator of SRF that can induce expression of SRF-dependent, smooth muscle-specific genes and actin/Rho-dependent genes, but not MAPK regulated growth response genes. How MRTFA and SRF discriminate between these sets of target genes is still unclear. We hypothesized that SWI/SNF ATP-dependent chromatin remodeling complexes, containing Brahma-related gene 1 (Brg1) and Brahma (Brm), may play a role in this process. Results from Western blotting and qRT-PCR analysis demonstrated that dominant negative Brg1 blocked the ability of MRTFA to induce the expression of smooth muscle-specific genes, but not actin/Rho-dependent early response genes. MRTFA over-expression did not induce expression of smooth muscle-specific genes in SW13 cells, which lack endogenous Brg1 or Brm. Reintroduction of Brg1 or Brm into SW13 cells restored their responsiveness to MRTFA. Immunoprecipitation assays revealed that Brg1, SRF and MRTFA form a complex in vivo and Brg1 directly binds MRTFA, but not SRF, in vitro. Results from chromatin immunoprecipitation assays demonstrated that dominant negative Brg1 significantly attenuated the ability of MRTFA to increase SRF binding to the promoters of smooth muscle-specific genes, but not early response genes. Together these data suggest that Brg1/Brm play a critical role in regulating expression of SRF/MRTFA-dependent smooth muscle-specific genes but are not required for SRF/MRTFA-dependent early response genes.

200. Chamber-specific enhancer analysis of zebrafish ventricular myosin heavy chain gene

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The two types of chambers in the vertebrate heart, the ventricles and the atria, have different contractile and electrophysiological properties, and express distinct sets of genes. However, the transcriptional regulation of these chamber-specific genes during development is still unclear. Here, we dissect the chamber specific enhancer of Zebrafish ventricular myosin heavy chain (vmhc), whose expression restricts to ventricle even before the heart becomes chamberized. We have isolated a 6 kb fragment from its 5' flanking sequence which can recapitulate the endogenous gene activity. Deletion analysis restricts the chamber-specific enhancer to a 2.2 kb fragment; however, a shorter 0.8 kb fragment drives GFP expression in the whole heart. Indeed, a series of deletion analysis identifies multiple sub-fragments within the 2.2 kb fragment that exhibit tissue-specific expression patterns in either ventricle or the whole heart. This study suggests that chamber-specific gene expression of zebrafish vmhc is controlled by a combination of multiple cis-acting elements.

201. Sorting Out The VCS Cells: A Window into Molecular and Cellular Signatures of the Developing His-Purkinje Network

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The mammalian cardiac conduction system, comprised of the SAN, AVN, and the fast-conducting His-Purkinje ventricular network, is modular in structure and in development. During embryogenesis, corresponding primordial cell populations, each with their unique molecular signature, are instructed to become patterned and specified before integrating into a functional unit driving apex-to-base contraction at around E13. As ventricular conduction system (VCS) cells mature, they exit the cell cycle before their working myocyte counterparts, eventually forming the bundle of His, bundle branches, and Purkinje fibers through differentiation, proliferation, and likely recruitment of neighboring myocytes. The electrically specialized VCS cells can be discerned from surrounding myocytes by the observed differential expression of ion channels and gap junctions in each population. We have found that the homeobox transcription factor Iroquois 3 (Irx3) is uniquely expressed in the developing and adult VCS, and is required for ventricular conduction function. Taking advantage of this developmentally relevant expression pattern, and the availability of an Irx3-eGFP BAC transgenic line, we have devised a FACS sorting strategy to isolate single GFP-positive VCS cells from non-fluorescent cells of the embryonic and neonatal mouse heart. qRT-PCR using primers specific to Irx3, TASK-1, Cx40, and Kv4.2 revealed their expected relative expression profiles in E14.5 GFP+ versus GFP- cells. Future experiments using this system at crucial VCS developmental time points followed by microarray profiling, TaqMan Low-Density Arrays, cell culture or single cell electrophysiology studies could prove to be invaluable in defining regulatory cues driving His-Purkinje formation. Moreover, when employed within the context of various mutations known to affect VCS function, this simple strategy could be used to address how VCS cells are specified, how they acquire specialized conduction properties, and allows for the assessment of the importance of transcriptional control.

202. The SWI/SNF chromatin remodeling complex is essential for myocardin-induced smooth muscle-specific gene expression

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Transcription regulatory complexes comprising myocardin and serum response factor (SRF) are critical for the transcriptional regulation of many smooth muscle-specific genes. However, little is known about the epigenetic mechanisms that regulate the activity of these complexes, within intact chromatin, during smooth muscle differentiation. In the current study we demonstrate that ATP-dependent chromatin remodeling enzymes, containing Brg1 or Brm1, are required for the myogenic activity of myocardin/SRF complexes. We found that the myogenic activity of myocardin is abrogated in cells expressing dominant negative Brg1. Furthermore, myocardin is unable to induce expression of smooth muscle-specific genes in SW13 cells, that lack endogenous Brg1 and Brm1, whereas reconstitution of Brg1 or Brm1 into these cells restored their responsiveness to myocardin. Brg1 directly binds to myocardin, forms a complex with SRF and myocardin in vivo and SWI/SNF complexes are required for myocardin to increase SRF binding to the promoters of smooth muscle-specific genes. Together, our data demonstrate that the ability of myocardin to induce smooth muscle-specific gene expression is dependent on its interaction with SWI/SNF ATP-dependent chromatin remodeling complexes. In addition, SWI/SNF recruitment plays an important role in determining which SRF-dependent promoters are activated by myocardin.

203. Myocardial expression of mutant p53 protects against doxorubicin-induced cardiomyocyte apoptosis and preserves heart function

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Doxorubicin, a topoisomerase II inhibitor that is commonly used to treat childhood cancer, induces p53-mediated cardiomyocyte apoptosis which can ultimately lead to heart failure. We hypothesized that cardiac-restricted expression of a dominant interfering p53 mutant (CB7) would protect cardiomyocytes from doxorubicin-induced apoptosis in vivo. Age-matched CB7 transgenic mice and control nontransgenic (NTG) mice were treated with a total of 20mg/kg of doxorubicin (2 intraperitoneal injections of 10mg/kg at 3-day interval). The prevalence of cardiomyocyte apoptosis was assessed via anti-activated caspase-3 immune reactivity. Cleavage of poly (ADP-ribose) polymerase (PARP, a substrate of caspase-3) and the level of the anti-apoptotic protein Bcl-xL were also monitored. Cardiac function was assessed using the two dimensional echocardiography. Our results showed that the prevalence of cardiomyocyte apoptosis was reduced in CB7 mice as compared to NTG mice following doxorubicin injection (activated caspase 3 immune reactivity: 0.85±0.14 positive cardiomyocytes/mm2 vs. 1.98±0.24 positive cardiomyocytes/mm2, $p<0.05$, $n=6$). In agreement with this, caspase-dependent PARP cleavage was not detected in the doxorubicin-treated CB7 hearts but was detected in the doxorubicin-treated NTG hearts. Bcl-xL expression was induced to a greater extent in CB7 mice as compared to NTG mice. Finally, fractional shortening was better preserved in the CB7 mice as compared to the NTG mice (7.79±1.35% reduction post-injection vs. 28.87±5.48%, $p<0.05$, $n=6$). Interestingly, the extent of functional preservation observed in doxorubicin-treated CB7 mice appeared to exceed that which would be expected based on the reduced levels of cardiomyocyte apoptosis. In conclusion, cardiac expression of CB7 protects against doxorubicin-induced cardiomyocyte apoptosis and preserves heart function. The anti-apoptotic effects of CB7 might result from the increased level of Bcl-xL. The disproportionate preservation of cardiac function suggests that antagonization of p53 may engender additional cardioprotective activity.

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