Qualitative and quantitative analyses of the morphological-dynamics of early cardiac pump action using video densidometry and optical coherence tomography (OCT)

Männer, Jörg; Thrane, Lars; Thommes, Jan; Happel, Christoph M.; Yelbuz, Mesud

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This program is supported in part by the American Heart Association's Council on Cardiovascular Surgery and Anesthesia

Table of Contents

Welcome to the Weinstein 2010 meeting in Amsterdam the Netherlands

On behalf of the organizing committee, we would like to welcome you to the 2010 Weinstein Cardiovascular Development Conference. Since its first rendezvous in 1994, the Weinstein conference has evolved into the largest annual meeting for scientists investigating the development of the cardiovascular system. We are proud to be able to organize the Weinstein Meeting, outside the United States, in the city of Amsterdam.

The conference building is part of the Colonial Institute, which was originally founded to promote trade and industry in the Dutch colonial territories. Today, the institute's role is more an educational one and the museum next door houses an anthropological collection illustrating its historical connections with the former colonies.

Amsterdam is Holland's largest city (roughly 750.000 inhabitants) although relatively small by international standards. Therfore, you will be pleasantly surprised to find a comfortably secure city center, with a lively atmosphere and relaxed feel about it. In fact, Amsterdam is one of the safest cities in the world. Even at night Amsterdam is a safe city to wander through and you will very rarely find yourself alone on the streets, even in the earliest hours. As in almost any bustling city in the world, pickpockets and petty thieves will take advantage of the careless, the best defense being simple common sense. Don't leave your bags and belongings unattended. Don't walk around with a lot of valuables. Don't put your valuables in your rucksack or shoulder bag hanging on your back, but keep them on you or in a money belt. Pay extra attention in crowded areas.

We are proud to announce that we have 383 participants this year, with 195 from Europe, 149 from North America, 35 from Asia, 2 from Australia, and 2 from Africa. From the 246 abstracts submitted, an expert panel of reviewers has selected [from blinded copies] 49 abstracts for platform presentation. As the presenters of many excellent abstracts could not be honored with a platform presentation, we expect to have three exciting and engaging poster sessions. The late afternoon session on Friday will have a more tutorial/educational emphasis, focusing on 3D image analysis en 3D presentation.

In contrast to other years we have two keynote speakers. Our first keynote speaker is Prof Denis Duboule. Prof Duboule is considered one of the world's leading specialists in developmental biology. His pioneering work on Hox gene clusters, revealing their basic mechanistic functions during developmental processes, launched forth a line of research which remains today one of the most intensively studied systems in biology. Our second keynote speaker Prof Frank Grosveld. Prof Grosveld has carried out pioneering work on many aspects of gene regulation from concluding that DNA methylation inhibits gene expression via an indirect mechanism, to the first description of a locus control region (LCR). Both topics are very pertinent to cardiovascular development.

On Saturday, as part of our evening's entertainment and to give you a different view of Amsterdam, we will embark on canal boats to see the city from water-level whilst enjoying cheese and wine. The boats will take us by canal to the party venue, the Olaf Chapel, which was built in 1440. Here we will eat and continue our evening with live music and dance.

We hope you will have a memorable and inspiring experience in Amsterdam.

The Local Organizing Committee
Maurice van den Hoff, Katherine Yutzey, Ruth van der Gaag, Antoon Moorman,
Alexandre Soufan, Phil Barnett, Vincent Christoffels, Alex Postma and Yigal Pinto

General Announcements and Information

Platform presentations
Oral presentations are limited to a total of 20 minutes: being **15 minutes presentation** at the most, followed by **5 minutes for questions and discussion**. Please do not exceed the allotted time. Your presentation should be uploaded at least 15 minutes before your session starts. All the presentations will be given in the great Hall. There are no parallel sessions.

Posters
All posters will be on display for one day only. There are 3 poster sessions. In the abstract book you will find the session in which your poster should be presented. Posters for session "A" should be presented on Thursday; those for session "B" should be presented on Friday and those for session "C" should be presented on Saturday.
The posters should be hung on the boards before the morning session of that day starts and should be taken down after the last afternoon session of that day.
Your abstract and its associated number will be posted on the boards to indicate where you should put your poster.

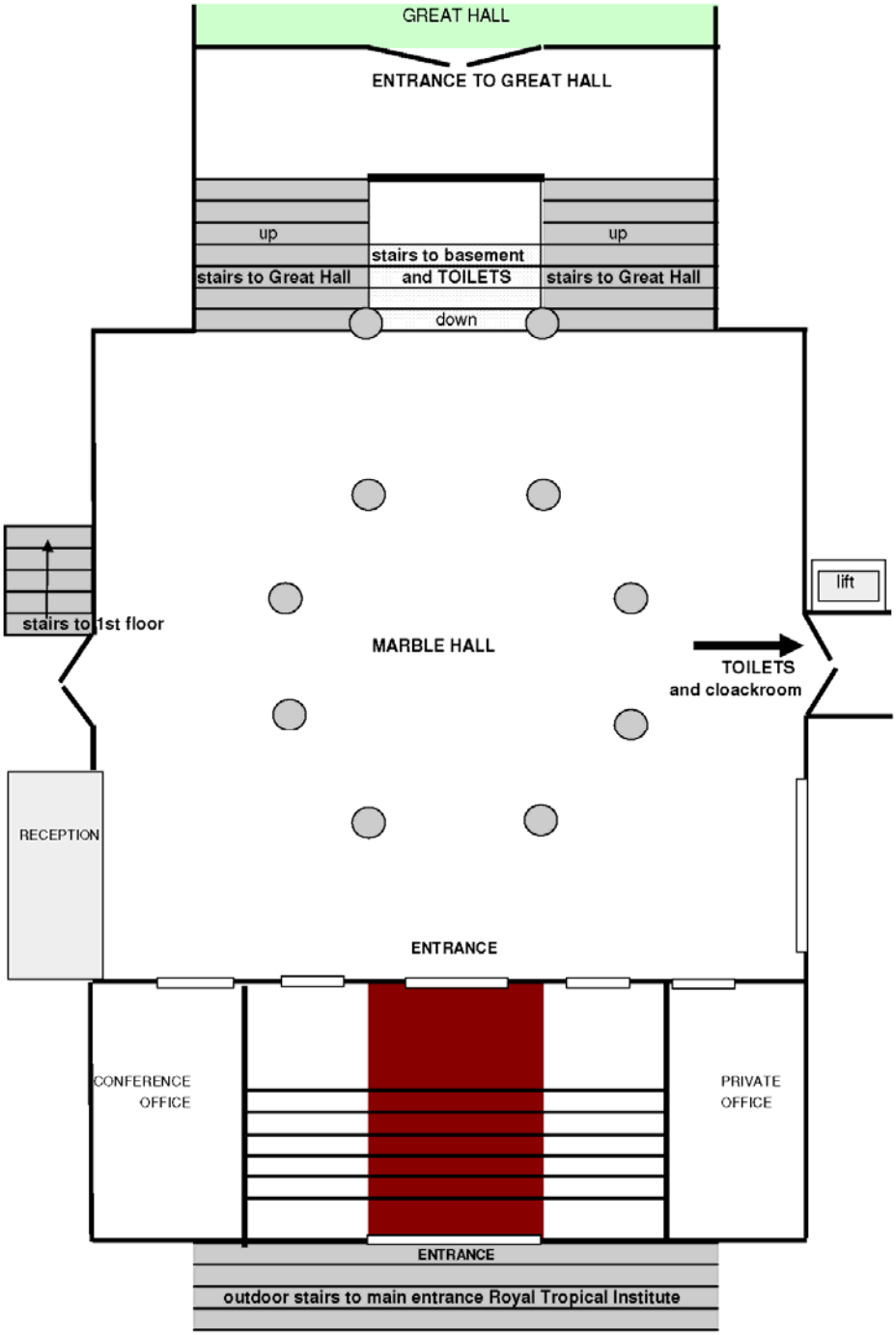
Catering
There will be morning coffee and tea, but no food from 8.00-9.00 before the morning sessions start, so please remember to eat breakfast before you come to the meeting.
During each break there will be coffee, tea and water and biscuits
For lunch an assortment of bread rolls with cheese, meat and other fillings will be available, which will be accompanied by coffee, tea, milk, butter milk, juice and water.
On Thursday evening there will be a buffet dinner in the Marble Hall of the Tropenmuseum.
Saturday evening's banquet will take place in Olaf Chapel of the Barbizon Palace hotel.
Tickets for all events and for 14 consumption tokens will be given when you register. Cash can also be payed for extra drinks at the bar.

Business Meeting
A business meeting to discuss future conference sites, amendments and modifications to the Weinstein Charter and selection of an ad hoc member to serve a 3-year term on the Weinstein Committee will be held in the Great Hall on Friday 21st May from 8.00-9.00.

Evaluations
Please fill out and return the evaluation form provided to you when you registered. These forms provide important feedback to help future organizers improve the Weinstein Cardiovascular Development Conference. These forms also provide information that is important for fundraising, which helps defray the cost of the meeting, allowing greater accessibility to the Conference by students and postdoctoral fellows.

Acknowledgements
We are very grateful to the many people at the Heart Failure Research Center, the Royal Tropical Institute and NH Hoteles who have worked so hard behind the scenes to make this meeting a success. We thank all the reviewers for the selection of anonymized abstracts for the oral presentations. We thank Prof Denis Duboule and Prof Frank Grosveld for serving as the Keynote Speakers this year. We also thank Dr. Constance Weinstein for her continued dedication to the mission of this meeting.

Floor plan Royal Tropical Institute



ROYAL TROPICAL INSTITUTE [TROPENMUSEUM]

Visiting address
Mauritskade 63
1092 AD Amsterdam

Central reception & information:
Telephone: + 31 20 568 8711
www.kit.nl

Condensed Schedule

Thursday 20 May 2010

8.00 - 10.00	**Registration** + tea/coffee + putting up posters for session A
10.10 - 10.20	**Welcome and Opening**
10.20 - 12.00	Platform session **TRANSCRIPTION REGULATION I**
12.00 - 14.00	Lunch + Poster session A
14.00 - 15.40	Platform session **TRANSCRIPTION REGULATION II**
15.40 - 16.20	Break
16.20 - 17.40	Platform session **CARDIAC PROGENITOR CELLS**
17.40 - 18.30	**KEYNOTE LECTURE by Prof Denis Duboule**:
18.30 - 19.30	**ESC-WG DAP Meeting**
18.30 - 20.00	Drinks
20.00 - 23.00	Buffet dinner + taking down of Posters of session A

Friday 21 May 2010

8.00 - 9.00	tea/coffee + putting up posters for session B
8.00 - 9.00	**Weinstein Business meeting**
9.00 - 10.40	Platform session **EPICARDIUM**
10.40 - 11.20	Break
11.20 - 13.00	Platform session **CARDIAC SIGNALLING**
13.00 - 14.30	Lunch + Poster session B
14.30 - 16.30	ESC-WG DAP session **GENETICS & CARDIOVASCULAR MALFORMATIONS**
16.30 - 17.00	Break + optional taking down of Posters of session B
17.00 - 18.40	Technical session **3D AND IMAGING TECHNIQUES**
18.45 -	Free evening + taking down of Posters of session B

Saturday 22 May 2010

8.00 - 9.00	tea/coffee + putting up posters for session C
8.00 - 9.00	**NIH presentation** Dr Charlene Schramm
9.00 - 11.00	Platform session **VALVE DEVELOPMENT**
11.00 - 11.45	Break
11.45 - 12.25	Platform session **HEART FIELDS**
12.25 - 13.05	**KEYNOTE LECTURE by Prof. Frank Grosveld**
13.05 - 14.30	Lunch + Poster session C
14.30 - 16.10	Platform session **NEURAL CREST & CONDUCTION SYSTEM**
16.10 - 16.40	Break + taking down of Posters of session C
16.40 - 17.40	Platform session **CARDIOMECHANICS**
17.40 - 17.50	Kathrine Yutzey Introducing the **Weinstein Conference 2011** in Cincinnati
17.50 - 18.00	Closure of the Scientific Part of the Meeting (Antoon? Maurice?)
18.00 -18.30	taking down of Posters of session C
18.30 - 20.00	"Floating wine and cheese party" Boat trip through the canals of Amsterdam to the Hotel Barbizon Palace
20.00 - 01.00	Farewell dinner party in the Olaf chapel (Hotel Barbizon Palace)

Weinstein Cardiovascular Development Conference Charter

Scope of the Conference

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

Organization of the Conference

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

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

Local Organizing Committee

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

Obligations of the Participants
One of the most important aspects of the Weinstein Conference has been the willingness of the participants to share new and unpublished information. This has provided opportunities for the participants to devise new experiments and develop new hypotheses in a collaborative manner. It is expected that all participants will participate in a collegial and ethical manner with respect to information obtained at the Weinstein Conference. Permission should be obtained before disclosure of another investigator's unpublished data.
Similarly, investigators pursuing similar experiments should inform a presenter if the divulged information has a bearing on their own work. All participants in the conference should be willing to share their expertise and reagents in the collective advancement of the area of cardiovascular development.

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

2010 Weinstein Members

Weinstein Committee (Term expiration indicated in parentheses)
Loren Field, Ph.D., Indiana University School of Medicine (2010)
Organizer, 2007 Weinstein Conference

James F. Martin, M.D., Ph.D., Texas A&M IBT, Houston (2011)
Organizer, 2008 Weinstein Conference

H. Joseph Yost, Ph.D., University of Utah (2011)
At-Large Member

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

Ray Runyan, Ph.D., University of Arizona (2012)
At-Large Member

Maurice van den Hoff, Ph.D, HFRC, Amsterdam (2013)
Organizer, 2010 Weinstein Conference

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

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

Local Committee Members 2010:
Maurice van den Hoff, Ph.D., Chair
Antoon Moorman, Ph.D., Co-Chair
Yigal Pinto, M.D. Ph.D., Co-Chair
Katherine Yutzey, Ph.D., Overseas Co-Chair
Ruth van der Gaag, Ph.D., Manager
Vincent Christoffels, Ph.D., Member
Alex Postma, Ph.D., Member
Phil Barnett, Ph.D., Member
Alexandre Soufan, Ph.D., Website Administrator

Weinstein Conference Host Institutions and Sites

1. Medical University of South Carolina (Charleston, 1994)
2. University of Rochester (Rochester, 1995)
3. University of Pennsylvania (Philadelphia, 1996)
4. University of Cincinnati (Cincinnati, 1997)
5. Vanderbilt University (Nashville, 1998)
6. University of Arizona (Tucson, 1999)
7. Washington University (St. Louis, 2000)
8. University of Texas, Southwestern Medical School (Dallas, 2001)
9. University of Utah (Salt Lake City, 2002)
10. Harvard University (Cambridge, 2003)
11. Leiden University (Leiden, Netherlands, 2004)
12. University of Arizona (Tucson, 2005)
13. University of South Florida (Tampa/St. Petersburg, 2006)
14. Indiana University School of Medicine (Indianapolis, 2007)
15. Texas A&M University (Houston, 2008)
16. University of California, San Francisco (San Francisco, 2009)
17. University of Amsterdam, Netherlands (Amsterdam, 2010)
18. University of Cincinnati (Cincinnati, 2011)
19. University of Chicago (Chicago, 2012)

2010 Weinstein Cardiovascular Development Conference
Keynote Speakers

Denis Duboule, PhD
Professor Federal Institute of Technology
Chair of the Department of Zoology and Animal Biology, University of Geneva
Chair for the national center of research 'Frontiers in Genetics' and current full professor at the Federal Institute of Technology (EPFL) in Lausanne.

Denis Duboule is considered one of the world's key specialists in developmental biology. His pioneering work on Hox gene clusters, revealing their basic mechanistic functions during developmental processes, launched forth a line of research which remains today one of the most intensively studied systems in biology.

Born in Geneva in 1955, Denis Duboule retains a dual French-Swiss nationality. Studying at the University of Geneva he received his doctorate of sciences in 1984. He then spent 10 years abroad, first at the medical faculty in Strasbourg (France), then at the European Laboratory for Molecular Biology (EMBL) in Heidelberg, Germany, as a group leader. In 1992 he took up the seat as full professor of Biology at the University of Geneva. A regularly feature guest author and editorial writer for Nature and Science, Denis Duboules original works, have to date been cited in excess of 10,000 times, top citation being entitled 'The structural and functional organization of the murine HOX gene family resembles that of Drosophila homeotic genes', published in the EMBO Journal in 1989. He has been a member of the Sciences-Cité Foundation's Board since 2000 and the chairman of Geneva's Cantonal Committee for Animal Protection since 2002. His honours and awards number many and include the Latsis Prize in 1994, the Louis-Jeantet Prize for Medicine in 1998, the Marcel Benoist Prize in 2003, and the French Academy of Science's Leopold Meyer Grand Prize in Biology in 2004. In 2005, he was awarded the Order of Chivalry in France's National Order of Merit (Ordre national du Mérite, founded by Charles de Gaulle, 1963).

Denis himself says he had a 'cool time at University' considering himself '... not a good example for real vocational science'. Although he may now reflect on his younger aspiration to be a sports teacher, the path he has chosen has certainly has allowed him to take a winning position in his track and field.

Selection of papers:

Papers cited more than 200 times:

Duboule D, Dollé P. (1989)The structural and functional organization of the murine HOX gene family resembles that of Drosophila homeotic genes. EMBO J. 8:1497-1505.

Dollé P, Izpisúa-Belmonte JC, Falkenstein H, Renucci A, Duboule D. (1989)Coordinate expression of the murine Hox-5 complex homoeobox-containing genes during limb pattern formation. Nature 342:767-772

Preitner N, Damiola F, Lopez-Molina L, Zakany J, Duboule D, Albrecht U, Schibler U. (2002) The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. Cell 110:251-60

Dollé P, Dierich A, LeMeur M, Schimmang T, Schuhbaur B, Chambon P, Duboule D. (1993) Disruption of the Hoxd-13 gene induces localized heterochrony leading to mice with neotenic limbs. Cell 75:431-441.

Izpisúa-Belmonte JC, Tickle C, Dollé P, Wolpert L, Duboule D. (1991) Expression of the homeobox Hox-4 genes and the specification of position in chick wing development. Nature 350:585-589.

Price M, Lemaistre M, Pischetola M, Di Lauro R, Duboule D. (1991) A mouse gene related to Distal-less shows a restricted expression in the developing forebrain. Nature 351:748-751.

Morgan BA, Izpisúa-Belmonte JC, Duboule D, Tabin CJ. (1992) Targeted misexpression of Hox-4.6 in the avian limb bud causes apparent homeotic transformations. Nature 358:236-239

Price M, Lazzaro D, Pohl T, Mattei MG, Rüther U, Olivo JC, Duboule D, Di Lauro R. (1992) Regional expression of the homeobox gene Nkx-2.2 in the developing mammalian forebrain. Neuron 8:241-255.

Duboule D, Morata G. (1994) Colinearity and functional hierarchy among genes of the homeotic complexes. Trends Genet. 10:358-64.

Frank Grosveld, PhD
Professor Erasmus University Rotterdam
Chair of the Department of Cell Biology

Frank Grosveld is Chair of Cell Biology at the Erasmus University Rotterdam, where "Mechanisms of gene regulation and epigenetic phenomena" have been the primary focus of his research. His group carried out pioneering work in many aspects of gene regulation from concluding that DNA methylation inhibits gene expression via an indirect mechanism, to the first description of a locus control region (LCR), visualizing the primary transcription process in the nucleus and most recently the description of 3D interactions in the nucleus. His group has filed a considerable number of patents and has closely collaborated with industrial partners.

Frank Grosveld studied biochemistry at the University of Amsterdam (MSc) and subsequently obtained a PhD from McGill University (1976) for his thesis work on the structure of bacteriophage S13. After two postdoctoral periods, one in Zurich under Charles Weissmann and the other in London at the National Institute for Medical Research under Richard Flavell. He went on to become Head of the division of Gene Structure and Expression at this NIMR in 1983. Since 1993 he is Chair of the Department Cell Biology at the Erasmus University Rotterdam, the Netherlands.

Frank Grosveld received the Jeantet prize for medicine in 1991 and the prestigious Spinoza prize in 1995. He is chairman of the Research School "Medical Genetic Centre" (MGC) and board member of the top research school "Centre for Biomedical Genetics" (CBG). He is and has been on the advisory board of many research councils, institutes and companies and is co-founder of four spin-off companies, he is scientific director of both Minos Biosystems Ltd and Harbour Antibodies BV.. He was recently appointed as Professor of the Royal Academy of Sciences (KNAW) in the Netherlands

Selection of papers:

Papers cited more than 200 times:

Rodriguez P, Bonte E, Krijgsveld J, Kolodziej KE, Guyot B, Heck AJ, Vyas P, de Boer E, Grosveld F, Strouboulis J (2005) GATA-1 forms distinct activating and repressive complexes in erythroid cells. EMBO J 6;24(13):2354-66

Meier N, Krpic S, Rodriguez P, Strouboulis J, Monti M, Krijgsveld J, Gering M, Patient R, Hostert A, Grosveld F (2006). Novel binding partners of Ldb1 are required for haematopoietic development. Development 133(24):4913-23

Jhunjhunwala S, van Zelm MC, Peak MM, Cutchin S, Riblet R, van Dongen JJ, Grosveld FG, Knoch TA, Murre C (2008).The 3D structure of the immunoglobulin heavy-chain locus: implications for long-range genomic interactions. Cell 133:265-79

de Laat W, Klous P, Kooren J, Noordermeer D, Palstra RJ, Simonis M, Splinter E, Grosveld F (2008). Three-dimensional organization of gene expression in erythroid cells. Curr Top Dev Biol 82:117-39

Monkhorst K, Jonkers I, Rentmeester E, Grosveld F, Gribnau J (2008). X inactivation counting and choice is a stochastic process: evidence for involvement of an X-linked activator. Cell 132:410-421

Places in Amsterdam to eat and drink

Since the places listed below are but a selection of what Amsterdam has to offer, here are some broader tips of areas in general.

The area immediately surrounding the Spui has many good restaurants and bars. The Zeedijk/Nieuwmarkt area has an oriental emphasis, but is a good place for lower budget meals and in general high quality.

An area to try and avoid is the Leidseplein. Although the food here is cheap and some places are ok, the area is a typical city tourist trap, a feature very much reflected in the quality of food served, which in general is pretty poor.

For those with a slightly larger budget and looking for a more up market area to dine we recommend checking out the area surrounding the 'Concertgebouw' which has some nice restaurants.

More information can be found on http://www.iamsterdam.com/en/visiting/visitingportal .

SOME FAVOURITE BARS IN AMSTERDAM, THOUGH NOT FOR TOO LARGE A GROUP

During the week most bars close at 1am, last orders at the bar are typically around 12:30am. On Friday & Saturday most places close later at around 2-3am.

Schutter	Typical student bar. Very friendly atmosphere http://www.deschutter.nl/
Beijaard	A good view of the trams as they speed by. Friendly bar, good beer, reasonable food http://www.beiaardgroep.eu/amsterdam/spui/spuhome/
Fonteijn	Popular bar, very mixed crowd. http://www.cafefonteyn.nl/
De Jaren	A large open bar, little bit pretentious, but has some nice views over the water http://www.diningcity.nl/cafedejaren/
t Smalle	A very 'Jordaneese' small bar. No music, but famous and popular http://www.t-smalle.nl/
Gollem	A nice Belgian type beer bar, serving over 200 different bottled beers. Small, but very cosy and popular http://cafegollem.nl/
de Zotte	A little gem behind the tourist trap of the Leidseplein. Good selection of beers and reasonable food http://www.dezotte.nl/
Eik en Linde	A very typical 'brown' cafe. Friendly, even though its full of locals. http://www.eikenlinde.nl/
Sluyswacht	A nice bar which is built on an old 'sluis' (lock-gate). Used to be the gate keepers house in the 17th century. http://www.sluyswacht.nl/
Heeren van Amstel	A very Dutch bar with live music late in the evenings. Friday and Saturday you will have to pay to get in after 10pm. www.deheerenvanaemstel.nl/

PLACES TO EAT

New King	http://www.newking.nl/	Chinese
Bird	http://www.thai-bird.nl/	Thai
Pakhuis	www.hetpakhuis.nl	Dutch
Rose's Cantina	http://www.dobson-uzcudun.com/mexican/roses_cantina.htm	Mexican
Humphreys	http://www.humphreys.nl/	Dutch
Kantijl Tijger	http://www.kantjil.nl/restaurant.htm	Indonesian
De Waag	http://www.indewaag.nl/	Dutch
Spargo	(opposite the Tropenmuseum) www.cafespargo.nl	Dutch
De Vijf Vliegen	http://www.thefiveflies.com	Dutch
Haesje Claes	http://www.haesjeclaes.nl	Dutch
Yam yam	www.yamyam.nl	Italian
Felicita	www.felicita.nl	Italian, no pizza

Weinstein 2010 sponsors

The Weinstein conference in Amsterdam would not have been possible without the financial support from:

Our platinum sponsors

HeartRepair; EU's 6th Framework Programme LSHM-CT-2005-018630

National Institutes of Health Research Grant # 5 R13 HL91580-03.
Funded by the National Heart, Lung, and Blood Institute and
the Eunice Kennedy Shriver National Institute of Child Health and Human Development.

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Invitrogen (Life Technologies)

Tebu-Bio

Detailed Schedule Weinstein Conference 2010

Great Hall of the Royal Tropical Institute, Amsterdam, the Netherlands

Thursday 20 May 2010

8.00 - 10.00	**Registration** + tea/coffee + putting up posters for session A
10.10 - 10.20	**Welcome and Opening** **Maurice van den Hoff** (Local organizing committee chair)

Platform session TRANSCRIPTION REGULATION I

Chairs: Phil Barnett & Tony Firulli

10.20 -10.40	**Conditional Disruption of Phosphoregulation of Hand1 Results in Outflow Tract Defects** Beth Firulli*, Joshua W. Vincetz, Ralston M. Barnes, Anthony B. Firulli
10.40 - 11.00	**Tbx2 and Tbx3 induce cushion formation and are required for AVC patterning** Reena Singh*, Willem M.H. Hoogaars, Thomas Grieskamp, Sameer Rana, Vincent M. Christoffels and Andreas Kispert
11.00 - 11.20	**The Cardiac Transcription Network Driven by the Interplay of Transcription Factors, Histone Modifications and microRNAs** Jenny Schlesinger*, Markus Schueler, Marcel Grunert, Jenny J. Fischer, Qin Zhang, Tammo Krueger, Martin Lange, Martje Tönjes, Ilona Dunkel, Silke R. Sperling
11.20 - 11.40	**A MEF2C-Myocardin complex directly regulates the cardiac metabolic master regulatory gene Ampk α2 through a conserved myocardial-specific enhancer** Courtney M. Anderson*, Analeah B. Heidt, Shan-Mei Xu, Brian L. Black
11.40 - 12.00	**Inhibition of cardiac myocyte apoptosis by Zac1, an essential cardiac transcription factor** Shinsuke Yuasa*, Takahiko Nishiyama, Owen W.J. Prall, Richard P. Harvey, Keiichi Fukuda
12.00 - 14.00	**Lunch + Poster session A**

Platform session TRANSCRIPTION REGULATION II

Chairs: Vincent Christoffels & Brian Black

14.00 - 14.20	**Hand2 loss-of-function in Hand1-expressing cells Reveals Distinct Roles In Heart And Coronary Vascular Development** Ralston M. Barnes*, Beth A. Firulli, Josh W. Vincentz, Peter Cserjesi, Simon J. Conway, Anthony B. Firulli
14.20 - 14.40	**Direct Reprogramming of Cardiac Fibroblasts into Functional Cardiomyocytes by Defined Factors** Masaki Ieda*, Jidong Fu, Vasanth Vedantham, Benoit G. Bruneau, and Deepak Srivastava

14.40 - 15.00	**Sox4 cooperates with Tbx3 in the regulation of T-box-gene targets** C.J.J. Boogerd*, L.Y.E. Wong, M. van den Boogaard, M.L. Bakker, P.A.C. 't Hoen, A.F. Moorman, V.M. Christoffels and P. Barnett.
15.00 - 15.20	**Prdm1, encoding the Blimp1 transcriptional repressor, genetically interacts with Tbx1 during distal outflow tract morphogenesis.** Stéphane D. Vincent*, Sachiko Miyagawa-Tomita and Margaret Buckingham
15.20 - 15.40	**A Direct microRNA Target Screen Reveals Alternate Regions of Complementarity that Mediate Repression in cardiac homeostasis** Eva Samal*, Vasanth Vedantham, Ru-Fang Yeh, Nathan Salomonis, YuanYuan Xiao, Morgan Von-Drehle, Linda van Laake, Yong Zhao, David J. Erle Deepak Srivastava
15.40 - 16.20	**Break**
Platform session CARDIAC PROGENITOR CELLS **Chairs: Christine Mummery & Scott Baldwin**	
16.20 - 16.40	**Endothelial progenitors contribute to the heart endocardium and play key roles in cardiac development** Michal Milgrom-Hoffman*, Napoleone Ferrara, Elazar Zelzer and Eldad Tzahor
16.40 - 17.00	**Identification of micro RNAs involved in cardiac specification in mESCs** Alexandre Colas*, Wesley McKeithan, Mark Mercola
17.00 -17.20	**Regulation of cardiac progenitor cells during heart development** Francesca Rochais* and Robert G. Kelly
17.20 - 17.40	**Hematopoietic cell contribution to embryonic mouse valve development and adult valve homeostasis** Zoltan Hajdu*, Roger R Markwald, Christopher C Drake, Imre Olah, Richard P Visconti
17.40 - 18.30	**KEYNOTE LECTURE** **Chair: Maurice van den Hoff** **Prof Denis Duboule**: **Genetic Control of Vertebrate Morphogenesis and Evolution** University of Geneva, Geneva & School of Life Sciences, Lausanne, Switzerland.
18.30 - 19.30	**ESC-WG DAP Meeting**
18.30 - 20.00	Drinks
20.00 - 23.00	Buffet dinner + taking down of Posters of session A

Friday 21 May 2010

8.00 - 9.00	tea/coffee + putting up posters for session B
8.00 - 9.00	**Weinstein Business meeting**

Platform session EPICARDIUM

Chairs: Thomas Brand & Takashi Mikawa

9.00 - 9.20	**Epicardial fate and Notch signaling** Thomas Grieskamp, Carsten Rudat, Julia Norden, Andreas Kispert*
9.20 - 9.40	**Loss of the Type III Transforming Growth Factor-β Receptor decreases Hyaluronic Acid responsiveness in Epicardial Cells** Nora S. Sánchez*, Evisabel Craig, Joseph D. Love, Todd Camenisch, and Joey V. Barnett
9.40 - 10.00	**NFATc1 in epicardium-derived cells is required for fibrous skeleton and intramyocardial coronary vessel development** Michelle D. Combs*, Caitlin M. Braitsch, and Katherine E. Yutzey
10.00 -10.20	**PDGF Signaling is Required for Epicardial Functions and New Blood Vessel Formation in Regenerating Zebrafish Hearts** Qiong Wu, Yolanda Zhang, Jieun Kim, Katie M. Wiens, Hiro Shimada, Robert I. Handin, Michael Y. Chao, Tai-Lan Tuan, Vaughn A. Starnes and Ching-Ling Lien*
10.20 - 10.40	**PDGFR signaling in the epicardium is required for epicardial EMT and coronary VSMC and cardiac fibroblast development** Christopher L. Smith*, Seung Tae Baek,and Michelle D. Tallquist
10.40 - 11.20	**Break**

Platform session CARDIAC SIGNALLING

Chairs: Paul Riley & Richard Harvey

11.20 - 11.40	**Inhibition of Bmp signaling by Smad6 is required for terminal differentiation of ventricular cardiomyocytes.** Emma de Pater and Jeroen Bakkers*
11.40 - 12.00	**The BMP Pathway Acts to Regulate Tbx20 in the Developing Heart** Erin Kaltenbrun*, Elizabeth Mandel, Thomas Callis, Da-Zhi Wang and Frank Conlon
12.00- 12.20	**Bmp-signaling regulates myocardial differentiation from cardiac progenitors through a micro RNA-mediated mechanism** Jun Wang*, Margarita Bonilla-Claudio, Jue Zhang, Yan Bai, Zheng Huang, Brian L. Black, Fen Wang, and James F. Martin
12.20 -12.40	**Cardiac Laterality is Controlled by Multiple Rho Kinase Signaling Pathways in Ciliated Cells** Guangliang Wang, Fiona C. Foley, Duck Soo Jang, Jeffrey D. Amack*
12.40 - 13.00	**Mitochondrial structure and function during cardiac myocyte differentiation** George A. Porter, Jr.*, Jennifer Hom, Rodrigo Quintanilla, David Hoffman, Bentley Karen, Shey-Shing Sheu
13.00 - 14.30	**Lunch + Poster session B**

ESC-WG DAP session GENETICS & CARDIOVASCULAR MALFORMATIONS
Chairs: Diego Franco & Alex Postma

14.30 - 14.50 **Dysregulation of the PDGFRA gene causes inflow tract anomalies including TAPVR: Integrating evidence from human genetics and model organisms**
Steven B. Bleyl*, Yukio Saijoh, Noortje A.M. Bax, Adriana C. Gittenberger-de Groot, Lambertus J. Wisse, Shigehito Yamada, Kohei Shiota and Gary C. Schoenwolf.

14.50 - 15.10 **Effects of conditional deletion of hypoxia-inducible factor (hif) and hypoxic stress on mouse heart morphogenesis**
Hongbin Liu and Steven A. Fisher*

15.10 - 15.30 **p53-related p63 transcription factor is essential for cardiac morphogenesis**
Alain Medawar, Matthieu Rouleau, Laurent Hamon, Huiqing Zhou, Hans van Bokhoven, Caterina Missero, Eleonora Candi, Cedric Blanpain, Gerry Melino, Michel Puceat and Daniel Aberdam*

15.30 - 15.50 **Copy Number variations (CNV) in left sided congenital heart disease**
Marc-Phillip Hitz*, Louis-Philippe Lemieux-Perreault, Christian R. Marshall, Bert Overduin, Maryse Thibeault, Alexandre F. R. Stewart, Stephen W. Scherer, Andrea Richter, Marie-Pierre Dubé, Gregor Andelfinger

15.50 - 16.10 **The role of FKbp12-notch1 mediated regulation in ventricular trabeculation and compaction**
Hanying Chen*, Wenjun Zhang, Xiao-Xin Sun, Zhuang Chen, Kryn Stankunas, Yongzhen He, Weidong Yong, Wuqiang Zhu, Simon Conway, Ching-Pin Chang, Loren J. Field, Nadia Carlesso, Weinian Shou

16.10 - 16.30 **MCTP2 is a novel gene causing aortic coarctation**
S.M. Ware*, X. Wang, L. Potocki, G. Zapata, M. Bray, A.C. Chinault, B.A. Boggs, E.K. Brundage, J.A. Towbin, A. Patel, S.D. Fernbach, S.L. Hamilton, K.L. McBride, J.W. Belmont, S.R. Lalani.

16.30 - 17.00 Break + taking down of Posters of session B

Netherlands Heart Foundation Technical session 3D AND IMAGING TECHNIQUES
Chairs: Jan Ruijter & Tim Mohun

17.00 - 17.20 **3D Modelling of heart development: looking to the future**
Tim Mohun

17.20 - 17.40 **Imaging in the Embryonic Heart: morphodynamic studies with Optical Coherence Tomography (OCT)**
Mesud Yelbuz

17.40 - 18.00 **How to quantify in 3D: 3D imaging of gene expression patterns and morphogenetic parameters in the embryonic heart.**
Jan M. Ruijter*, Alexandre T. Soufan, Jaco Hagoort, and Antoon F.M. Moorman

18.00 - 18.20 **How to place a section: automated fitting of histological sections into a high resolution 3D reference model of a developing mouse heart**
Bouke A de Boer*, Frans PJM Voorbraak, Maurice JB van den Hoff, Antoon FM Moorman, Jan M Ruijter

18.20 - 18.40 **How to make a 3D pdf: from 3D-reconstruction to interactive 3D-pdf; a protocol for biologists.**
Jaco Hagoort*, Alexandre Soufan, Bouke de Boer, Antoon Moorman

18.45 - Free evening + taking down of Posters of session B

Saturday 22 May 2010

8.00 - 9.00	tea/coffee + putting up posters for session C
8.00 - 9.00	**NIH presentation** Dr Charlene Schramm
Platform session VALVE DEVELOPMENT **Chairs: Katherine Yutzey & Andy Wessels**	
9.00 - 9.20	**MiR-23 is essential to restrict endothelial-to-mesenchymal transition during cardiac valve formation.** Anne Karine Lagendijk*, Jeroen Bakkers
9.20 - 9.40	**Cardiac valve malformations: new insights from Pdlim7, an unexpected suspect in heart development** Jennifer Krcmery*, Rudyard Sadleir, Rajesh Gupta, Chrissy Kamide, Sol Misener, Doug Losordo, and Hans-Georg Simon
9.40 - 10.00	**Krox20 is required during valve remodeling and maturation** Sarah Arab, Frank Kober, Piotr Topilko, Monique Bernard, Patrick Charnay, Patrick Cozzone and Stéphane Zaffran*
10.00 -10.20	(**The Metalloprotease ADAMTS5 Plays A Critical Role In ECM Stratification And Cusp Sculpting During Semilunar Valve Maturation** Christine B. Kern*, Daniel R. McCulloch, Jessica D. McGarity, Alexandra Bahan, Loren E. Danese, Courtney M. Nelson, Andy Wessels, and Suneel S. Apte
10.20 - 10.40	**Induction of a developmental gene program in pediatric and adult aortic valve disease** Elaine Wirrig*, Jonathan Cheek, Christina Alfieri, Walter Merrill, Robert Hinton, Katherine Yutzey
10.40 - 11.00	**Reduced Sox9 function promotes heart valve calcification in vivo** Jacqueline D Peacock*, Agata K Levay, and Joy Lincoln
11.00 - 11.45	**Break**
Platform session HEART FIELDS **Chairs: Margaret Buckingham & Jim Martin**	
11.45 - 12.05	**Tbx5 is required in the Second Heart Field for Atrioventricular Septation** Linglin Xie, Joshua M. Friedland-Little, Andrew D. Hoffmann, and Ivan P. Moskowitz*
12.05 - 12.25	**Cooperative roles of Tbx1 and Tbx3 during early outflow tract development** Karim Mesbah*, M. Sameer Rana, Laure Lo Ré, Virginia Papaioannou, Vincent M. Christoffels, Robert G. Kelly
12.25 - 13.05	**KEYNOTE LECTURE** **Chair Yigal Pinto** **Prof. Frank Grosveld: Interactions in the genome** *Dept of Cell Biology, Erasmus Medical Center, Rotterdam, the Netherlands*
13.05 - 14.30	**Lunch + Poster session C**

Platform session NEURAL CREST & CONDUCTION SYSTEM

Chairs: Margaret Kirby & Ivan Moskowitz

14.30 - 14.50	**Trigenic neural crest-restricted Smad7 over-expression results in congenital craniofacial and cardiovascular defects** Sunyong Tang, Paige Snider and Simon J. Conway *
14.50 - 15.10	**Cardiac Neural Crest Nibble Their Way to the Heart** Mary R. Hutson*, Ann Marie Scholl, Elizabeth B. Kuhn, Asako Sato, Harriett A. Stadt, Jennifer R. Decker, Kelly Pegram, and Margaret L. Kirby *Department of Pediatrics, Neonatal-Perinatal Research Institute, Duke University Medical Center, Durham, NC, USA
15.10 - 15.30	**Origins of Cardiac Pacemaking Cells in Avians** Michael Bressan*, Alicia Navetta, Takashi Mikawa
15.30 - 15.50	**The role of Tbx3 and Nkx2-5 in the adult atrioventricular conduction system** Mathilda T.M. Mommersteeg*, Bas J. Boukens, Saskia van der Velden, Corrie de Gier-de Vries, Richard P. Harvey, Antoon F.M. Moorman, Vincent M. Christoffels
15.50 - 16.10	**Delayed atrioventricular conduction in adrenergic-deficient embryonic mouse hearts: Uncovering a role for retinoic acid** Kingsley Osuala, David G. Taylor, Celines Martinez, Ha-Long Nguyen, Tu-Suong Nguyen, Anupama Natarajan, Peter Molnar, James Hickman, and Steven N. Ebert*
16.10 - 16.40	**Break + taking down of Posters of session C**

Platform session CARDIOMECHANICS

Chair: Roger Markwald & Ray Runyan

16.40 - 17.00	**Shear stress modulates transcriptional regulation and cushion endoMT in the developing heart** Beerend P. Hierck*, Anastasia D. Egorova, Simone van de Pas, Marie José Goumans, Peter ten Dijke, Robert E. Poelmann
17.00 - 17.20	**Integrative computational modeling and imaging approach to study the effects of abnormal hemodynamic conditions on cardiac development.** Sandra Rugonyi*, Ruikang Wang and Kent Thornburg
17.20 - 17.40	**Knockdown of embryonic myosin heavy chain causes defective electrical activities and calcium signalling within the developing heart** Catrin Rutland, Luis Polo-Parada, Aziza Alibhai, Aaran Thorpe and Siobhan Loughna*
17.40 - 17.50	Kathrine Yutzey Introducing the **Weinstein Conference 2011** in Cincinnati
17.50 - 18.00	Closure of the Scientific Part of the Meeting
18.00 -18.30	Taking down of Posters of session C
18.30 - 20.00	"Floating wine and cheese party" Boat trip through the canals of Amsterdam to the Hotel Barbizon Palace
20.00 - 01.00	Farewell dinner party in the Olaf chapel (Hotel Barbizon Palace)

ORAL SESSIONS
THURSDAY 20 MAY

Location: Great Hall

10.20 - 12.00 Transcription Regulation I
14.00 - 15.40 Transcription Regulation II
16.20 - 17.40 Cardiac Progenitor Cells
17.40 - 18.30 Keynote Lecture I

Transcription Regulation I

136. Conditional Disruption of Phosphoregulation of Hand1 Results in Outflow Tract Defects

Beth Firulli*, Joshua W. Vincetz, Ralston M. Barnes, Anthony B. Firulli
Riley Heart Research Center, Wells Center for Pediatric Research, Division of Pediatric Cardiology, Indiana Medical School

The Twist family of bHLH transcription factors are broadly expressed within mesenchymal cell populations and convey tissue specific transcriptional output to numerous organ systems such as the heart and neural crest-derived cardiac outflow tract. We hypothesize that dimer choice regulation conveys tissue-specific function by regulating the bHLH dimer pool within the cell. Dimer regulation is in part controlled by phosphoregulation of evolutionarily conserved threonine and serine residues within Helix 1 of the HLH domain. Indeed mutations that hobble phosphoregulation of Twist1 are causative to the human disease Saethre Chotzen Syndrome and involve a dimer modulation relationship with Hand2. To begin to understand more clearly how phosphoregulation of Twist-proteins affects biological outputs, we have conditionally targeted both hypo-phosphorylation and phosphorylation mimic alleles for Hand1. Activating the of expression of these Hand1 point mutant alleles within the neural crest via intercross with the Wnt1-cre driver results in cardiac outflow tract defects that result in neonatal death. Surprisingly, the observed phenotypes are more severe then neural crest specific ablation of Hand1, indicating that a compound heterozygous mouse with a single wildtype and complementary phospho-mutant Hand1 allele is more deleterious to survival then a neural crest Hand1 conditionally null mouse.

137. Tbx2 and Tbx3 induce cushion formation and are required for AVC patterning

Reena Singh* (1), Willem M.H. Hoogaars (2), Thomas Grieskamp (1), Sameer Rana (2), Vincent M. Christoffels (2) and Andreas Kispert (1)
(1) Institut für Molekularbiologie, Medizinische Hochschule Hannover, Hannover, Germany (2) Heart Failure Research Center (T.H., V.W., A.F.M.M., V.M.C.), Academic Medical Center, Amsterdam, The Netherlands.

The complex four-chambered heart is an outcome of regionalization, patterning and differentiation during embryonic development. In the developing heart, chamber myocardium of atrium and ventricles are separated and bordered by non-chamber myocardium of the atrioventricular canal (avc) and outflow tract (oft). The avc myocardium is required for aligning the atrial and ventricular chambers and for mesenchymal cushion formation. Cushion tissues are subsequently deployed in the formation of valves and septa. Work from the last decade has shown that T-box proteins, which are expressed in different compartments of the developing heart in exclusive and sometimes overlapping fashion, are downstream effectors of several signaling morphogens, particularly BMPs, and play crucial role in cardiac regionalization. Tbx2 is expressed in and required for the formation of the avc (Harrelson 2004; Aanhaanen 2009). However, Tbx2 mutants showed variable phenotypes with incomplete penetrance. Since the closely related paralog Tbx3 is co-expressed with Tbx2 in the avc, we generated and analyzed Tbx2/Tbx3 double mutants in order to eliminate the effect of redundancy. Our analysis indeed suggested that Tbx2 in combination with Tbx3 is crucial for avc establishment by suppressing chamber myocardial gene programs in this particular domain of the heart. Simultaneously, we also observed the insufficiency of double mutants to maintain Bmp2 expression and to induce cardiac jelly, the very first step of cushion formation. By gain of function studies we could show that Tbx2 and Tbx3 are individually sufficient for inducing cushion formation by activating Bmp2 and thereby acting in a feed-forward loop.

138. The Cardiac Transcription Network Driven by the Interplay of Transcription Factors, Histone Modifications and microRNAs

Jenny Schlesinger* (1), Markus Schueler (1,2), Marcel Grunert (1), Jenny J. Fischer (1), Qin Zhang (1), Tammo Krueger (1), Martin Lange (1), Martje Tönjes (1), Ilona Dunkel (1), Silke R. Sperling (1)
(1) Group Cardiovascular Genetics, Department of Vertebrate Genomics, Max Planck Institute for Molecular Genetics, Germany (2) Department of Computational Molecular Biology, Max Planck Institute for Molecular Genetics, Germany

Heart development is controlled by an evolutionarily conserved transcription factor network connecting signaling pathways with respective genes. In addition to the transcriptional machinery, epigenetic and post-transcriptional mechanisms contribute to the establishment of cardiac cell fates. We present a systems-level study integrating mRNA profiles with DNA-binding events of transcription factors (Gata4, Mef2a, Nkx2.5 and Srf), activating histone modifications (H3ac, H4ac, H3K4me2 and H3K4me3) and microRNA profiles obtained in wildtype and RNAi mediated knockdown. We confirmed conclusions primarily obtained in cardiomyocyte cell culture in a time course of cardiac maturation in mouse around birth. The high-throughput data have been generated using microarrays and next generation sequencing technology, whereas confirmation experiments were performed by luciferase reporter gene assays and quantitative real-time PCR. We provide insights for the combinatorial regulation by transcription factors and show that they partially compensate each other's function. Further, histone 3 acetylation has a significant impact on the regulation of Srf and Gata4 dependent genes, and moreover buffers the downregulation of Srf target gene expression in its knockdown. In accordance with others, we found that a high proportion of differentially expressed genes in RNAi experiments are indirect targets. On the example of Srf, we show that 43% of the altered mRNA profile can be explained by the impact of microRNA alterations. In summary, we show that the different levels regulating cardiac mRNA profiles, such as DNA-binding transcription factors, histone modifications and microRNAs, have an high degree of interdependency and the potential to buffer each other.

139. A MEF2C-Myocardin complex directly regulates the cardiac metabolic master regulatory gene Ampk α2 through a conserved myocardial-specific enhancer

Courtney M. Anderson*, Analeah B. Heidt, Shan-Mei Xu, Brian L. Black
Cardiovascular Research Institute, University of California, San Francisco, USA

The MADS domain transcription factor MEF2C is essential for cardiovascular development in mice. During embryogenesis, Mef2c is expressed at the onset of cardiac muscle differentiation, and Mef2c-null embryos die by E10 with severe cardiac morphogenetic and differentiation defects. MEF2C is a signal-dependent transcription factor that can function as either an activator or a repressor, depending on cofactor interactions. Myocardin and Myocardin-related transcription factors are SAP domain proteins that are potent coactivators for the MADS domain transcription factor SRF. Recently, a specific splice isoform of Myocardin, Myocardin-935, has been identified as a potent coactivator of MEF2C activity, yet the targets of MEF2C-Myocardin and the transcriptional mechanisms that control Myocardin coactivation of MEF2C remain unresolved. In this study, we identified a highly conserved transcriptional enhancer from the Ampka2 gene that is sufficient to direct expression exclusively to the myocardium during development and in adulthood. Ampka2 encodes a subunit of the 5'-AMP-activated protein kinase (AMPK), an energy-sensing enzyme that maintains cellular energy homeostasis and controls cardiac metabolism by regulating fatty acid oxidation, glycolysis, and glucose uptake. We show that the Ampka2 myocardial enhancer requires a MEF2C-Myocardin complex for activity in vivo and in vitro, and that this complex regulates Ampka2 expression through two essential, highly conserved MEF2 sites in the enhancer. Importantly, these results are the first to demonstrate the transcriptional regulation of Ampka2 in the heart in vivo, and we have identified a general mechanism by which MEF2C and Myocardin form a complex and regulate transcription of cardiac genes.

140. Inhibition of cardiac myocyte apoptosis by Zac1, an essential cardiac transcription factor

Shinsuke Yuasa*, Takahiko Nishiyama, Owen W.J. Prall, Richard P. Harvey, Keiichi Fukuda
Department of Cardiology, Department of Internal Medicine Center for Integrated Medical Research, Keio University School of Medicine Victor Chang Cardiac Research Institute University of New South Wales

The transcriptional mechanisms are poorly understood in the heart, despite the identification of several essential cardiac transcription factors. We found that Zac1, which encodes a zinc-finger-type transcription factor and is a maternal imprinting gene, was strongly expressed throughout the murine heart from embryonic stage 8.5. Zac1 was a potent activator of several cardiac genes and bound directly to the atrial natriuretic peptide promoter, upon which Zac1 exerted a strong synergistic transcriptional activity and a physical interaction with Nkx2.5. Nkx2.5 also activated the Zac1 promoter, and Nkx2.5-null hearts showed decreased Zac1 expression. Zac1-mutated mice showed decreased levels of several cardiac genes and severe cardiac malformation. The absence of Zac1 expression resulted in increasing the number of apoptotic cells in the heart. These data indicate that Zac1 is an essential cardiac transcription factor that acts both independently and co-operatively with Nkx2.5.

Transcription Regulation II

141. Hand2 loss-of-function in Hand1-expressing cells Reveals Distinct Roles In Heart And Coronary Vascular Development

Ralston M. Barnes*(1), Beth A. Firulli(1), Josh W. Vincentz(1), Peter Cserjesi(2), Simon J. Conway(1), Anthony B. Firulli (1)
(1) Riley Heart Research Center, Wells Center for Pediatric Research, Division of Pediatric Cardiology, Indiana Medical School (2) Department of Cell & Molecular Biology, Tulane University, New Orleans, LA, USA

Hand1 and Hand2, members of the Twist-family of bHLH transcription factors, are expressed in a partially overlapping and complimentary profile in the developing heart. Hand2 expression persists throughout the entire linear heart tube and then downregulated within the left ventricle at the onset of cardiac looping. In contrast, Hand1 is expressed at the arterial and venous poles of the linear heart tube at E8.0-8.5 becoming robust within the developing left ventricle and outflow tract. To investigate Hand factor function in the heart, we engineered a Hand1 allele expressing Cre Recombinase for lineage analysis. We show that cardiac Hand1 expression is largely limited to cells of the primary heart field revealing little temporal overlap with Hand2. Surprisingly, we identify transient Hand1 expression domain within the septum transversum that marks the future proepicardium, epicardium, and derivatives. To look precisely at coexpression of Hand1 and Hand2, we conditionally delete Hand2 from Hand1-expressing cells (H2CKO). H2CKOs die at E14.5. Defects include oligodactyly and syndactlyly of the digits, persistent truncus arteriosis and double outled right ventricle of the outflow tract that is attributed to defective PDGF signaling, and abnormal cardiovascular maturation. Strikingly, we observe Hand2 deletion from the proepicardium, which results in faulty epicardialization, impaired Fn1 fibril assembly, and leads to a failure to establish coronary arteries. Ablation of Hand2 within the Wt1-lineage recapitulates observed epicardial defects and time of death. Together, these data show a hierarchal relationship where transient Hand1 expression within the septum transversum defines epicardial precursors that are dependent on Hand2 function.

142. Direct Reprogramming of Cardiac Fibroblasts into Functional Cardiomyocytes by Defined Factors

Masaki Ieda* (1,2,3), Jidong Fu (1,2,3), Vasanth Vedantham (1,2,3), Benoit G. Bruneau (1,2,4), and Deepak Srivastava (1,2,3)
(1)Gladstone Institute of Cardiovascular Disease; (2)Department of Pediatrics; (3)Department of Biochemistry and Biophysics, (4)Cardiovascular Research Institute, University of California, San Francisco, CA 94158, USA.

The reprogramming of fibroblast cells to induced pluripotent stem (iPS) cells by a combination of defined factors demonstrated that somatic cells can be induced to alter their cell fate. A large pool of fibroblast cells exists in the post-natal heart, yet no single “master regulator” of cardiac reprogramming has been identified. Here, we report that a combination of three developmental transcription factors rapidly and efficiently reprogrammed post-natal cardiac fibroblasts directly into differentiated cardiomyocytes. Induced cardiomyocytes expressed cardiac-specific markers, had a global gene expression profile similar to cardiomyocytes, and contracted spontaneously. Fibroblast cells transplanted into mouse hearts one day after transduction of the three factors differentiated into cardiomyocytes in vivo. These findings demonstrate that functional cardiomyocytes can be directly reprogrammed from differentiated somatic cells by defined factors. Reprogramming of endogenous or explanted cardiac fibroblast cells might provide a source of cardiomyocytes for regenerative approaches.

143. Sox4 cooperates with Tbx3 in the regulation of T-box-gene targets

C.J.J. Boogerd* (1), L.Y.E. Wong (1), M. van den Boogaard (1), M.L. Bakker (1), P.A.C. 't Hoen (2), A.F. Moorman (1), V.M. Christoffels (1) and P. Barnett (1).
(1) Heart Failure Research Centre, Academic Medical Centre, University of Amsterdam, Meibergdreef 15, 1105AZ Amsterdam, The Netherlands. (2) Center for Human and Clinical Genetics, Leiden University Medical Center, P.O. Box 9600, 2300 RC Leiden, The Netherlands.

Tbx3 plays essential roles during early development, regulating key steps in development of the heart and other organ systems. To gain insight into the molecular aspects of the function of Tbx3, we employed the two-hybrid system to identify novel protein interaction partners of Tbx3. From this screen we identified the HMG-box containing transcription factor Sox4. We found that the patterns of expression of Tbx3 and Sox4 during cardiac development display considerable overlap, indicating potential sites of functional interaction. Using GST pulldown assays and a nuclear colocalization study, we verified the direct protein interaction. By performing genome wide Tbx3 ChIP-seq studies in mouse hearts and comparing this data to Sox4 ChIP-chip studies, we identified putative downstream gene targets of this interaction. Several of these promoter fragments were shown to be bound by Tbx3 and Sox4 by performing ChIP assays on embryonic mouse heart. We identified a possible role for these proteins in the cooperative regulation of Gja1 encoding Cx43 gap junction protein, via a novel enhancer positioned in the intron of Gja1, providing new molecular insights into the regulation of this key cardiac conduction protein.

144. Prdm1, encoding the Blimp1 transcriptional repressor, genetically interacts with Tbx1 during distal outflow tract morphogenesis.

Stéphane D. Vincent* (1), Sachiko Miyagawa-Tomita (2) and Margaret Buckingham (1)
(1) Institut Pasteur, Departement of Developmental Biology, Paris, France (2) Tokyo Women's Medical University, Pediatric Cardiology, Tokyo, Japan

The Prdm1/Blimp1 protein acts as a transcriptional repressor by recruiting co-repressors leading to direct repression and indirect activation of gene expression. Prdm1 is dynamically expressed during development and, strikingly, has been shown to control specific differentiation programs in each of the different domains studied to date. During heart formation, Prdm1 is expressed in the second heart field (SHF). In this context, like Tbx1, Prdm1 is also expressed in the adjacent endoderm where it is required to support the growth of the mesenchymal cells of the branchial arches 2 to 6. As a result, Prdm1-/- mutant embryos display heart septation defects. In order to analyse a cell-autonomous function in the SHF, we carried out a conditional deletion of Prdm1 using MesP1Cre (early cardiac mesoderm) and Mef2cCre (anterior SHF) lines. Mesp1Cre mutants die at birth and display defects in structures of the distal OFT, derived from the SHF: uneven semilunar valves, misalignment of the great arteries and aortic arch patterning defects. Mef2cCre conditional mutants are viable, but display also mild aortic arch patterning defects. Our results suggest that Prdm1 plays an essential early regulatory role in controlling the proliferation of mesodermal progenitor cells of the SHF that contribute to morphogenesis of the arterial pole of the heart. The expression profiles and the mutant phenotypes of Prdm1 and Tbx1 are very similar, suggesting that these genes could be acting in the same pathway during heart development. We show here that Prdm1 and Tbx1 genetically interact during arterial pole development.

145. A Direct microRNA Target Screen Reveals Alternate Regions of Complementarity that Mediate Repression in cardiac homeostasis

Eva Samal* (1, 4), Vasanth Vedantham (1,2), Ru-Fang Yeh (3), Nathan Salomonis (1), YuanYuan Xiao (3), Morgan Von-Drehle (1), Linda van Laake (5), Yong Zhao (6), David J. Erle (2) Deepak Srivastava (1,4)
(1) Gladstone Institute of Cardiovascular Disease and Departments of Biochemistry and Biophysics (2) Medicine (3) Biostatistics (4) Pediatrics, University of California, San Francisco, CA, 94158, (5) University of Utrecht, The Netherlands. (6) Department of Genetics and Genomic Sciences, Mt Sinai School of Medicine, NY, USA

Distinct signature patterns of microRNAs (miRNAs) in healthy versus diseased hearts combined with their ability to regulate specific aspects of cardiac function offer precedence for using miRNAs as therapeutic agents for heart dysfunction. However, accurate determination of miRNA targets is a prerequisite before miRNA-based approaches can be employed in the management of heart disease. This has remained a challenge due to imperfect miRNA::mRNA base-pairing. Bioinformatic approaches based on sequence complementarity among the first eight nucleotides of miRNAs known as a “seed” sequence have been useful but cannot account for all phenotypes observed with dysregulation of miRNA dosage. Here we describe an unbiased direct experimental screen for miRNA target identification. Using this screen, we validated numerous novel miR-1 interacting cardiac mRNAs including those with canonical 5′ seed matches and several with imperfect seed matches. Surprisingly, our screen also revealed a subset of mRNA targets with very limited 5′ base-pairing but extensive complementarity to the mid-portion of the miRNA. This region of alternate complementarity was required for miRNA mediated repression. The novel targets were validated in vivo and correlated with in vivo function of miR-1 thus providing a molecular basis for perturbations in cell cycle control, cardiogenesis and cardiac conduction abnormalities such as long QT syndrome and associated torsades de pointes observed with misregulation of miR-1 levels. The experimental strategy described here offers an efficient approach to identify physiologically relevant miRNA targets and also reveal an alternate region of complementarity that could direct miRNA-mediated repression of gene expression.

Cardiac Progenitor Cells

92. Endothelial progenitors contribute to the heart endocardium and play key roles in cardiac development

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The primitive heart is composed of two cell layers, the myocardium, which contributes to cardiac muscle tissue, and the endocardium, which covers the inner lumen of the heart. While significant progress has been made towards elucidating the embryonic origins of the myocardium, the embryonic origins of the endocardium are far less clear. Myocardial and endocardial progenitors are thought to arise from both uni- and multipotent cardiovascular progenitors within the cardiac crescent. Here, using cellular and molecular analyses in chick embryos, we identified an endocardial-forming field residing outside the cardiac crescent, in a continuum with the endothelial plexus. Strikingly, embryonic endothelial cells implanted into the cardiac environment specifically contributed to the endocardium. To further investigate the endothelial origins of the endocardium, and the necessity of endothelial progenitors for heart development in the mouse, we conditionally ablated vascular endothelial growth factor receptor 2 (Flk1) in cardiac and endothelial progenitors. Ablation of Flk1 in both cardiac and endothelial mesoderm progenitors using MesP1Cre led to complete loss of the endocardium, as well as broader defects in second heart field derivatives. In contrast, the endocardium was not affected upon ablation of Flk1 only within the cardiac crescent using Isl1Cre, suggesting that Flk1 expression in Isl1+ cardiovascular progenitors has a minor impact on the endocardial cell lineage in vivo. Collectively, our findings suggest that the endocardium derives from an endothelial origin, and that endothelial progenitors play key roles in the morphogenesis of the heart.

93. Identification of micro RNAs involved in cardiac specification in mESCs

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MicroRNAs (miRs) are naturally-occurring, small non-coding RNAs that regulate many aspects of cell biology by influencing the stability and translation of target mRNAs. Recent studies have highlighted their importance during cardiogenesis. However, defining the precise roles they play has been limited by the difficulty of performing large-scale functional studies in higher vertebrate embryos. In this study, we have developed a mouse embryonic stem cell (mESC)-based cardiogenesis miR screen in defined media. mESC cultures were differentiated to induce uncommitted mesoderm (day 3) and transfected with a library of 328 miRs and cardiogenesis was quantified by measuring the fluorescence of eGFP under the control of the cardiac specific a-MHC promoter. Four families of miRs showed a robust ability to induce cardiogenesis in mESCs. Among them, the miR-18 family displayed the most potent pro-cardiac activity. In order to gain insight into the molecular mechanism of action, we tested whether validated or TargetScanMouse5.1-predicted mRNA targets could phenocopy the cardiogenic action of miR-18. Smad2 is a predicted target of miR-18 that is conserved across vertebrates. Smad2-siRNA-mediated knockdown was found sufficient to induce cardiogenesis at similar levels to miR-18, while the knockdown of two validated targets (Runx1, ESR1) alone or in combination did not. Moreover we showed that miR-18 is able to bind a Smad2-3'UTR-GFP sensor and induce its degradation and down-regulate Smad2 mRNA in differentiating mESCs. Altogether, these results suggest that Smad2 is likely to be the principal relevant target of miR-18 involved in enhancing cardiogenesis. Attenuation of the TGFb/Nodal pathway, after mesoderm and endoderm induction has occurred, has been correlated with enhanced cardiogenesis in mESCs. However, the biological processes that leads to the corresponding increase in cardiogenesis is not fully understood. Here we show that in both miR-18 gain of function and Smad2 siRNA-mediated knockdown, Flk-1 expression was dramatically up-regulated two days after transfection, leading to the subsequent up-regulation of several cardiac specific makers (Nkx2.5, Isl-1, MEF2C, GATA-4) at day 6. Altogether these data suggest that miR-18 attenuates TGFb/Nodal signaling via Smad2to specifying Flk-1+ cardiovascular progenitors

94. Regulation of cardiac progenitor cells during heart development

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Cardiac progenitor cells of the second heart field (SHF) contribute to the poles of the elongating embryonic heart and express genes encoding the fibroblast growth factor Fgf10 and transcription factor Isl1. Perturbations of SHF development lead to congenital heart defects. Recent studies have demonstrated the existence, in the later heart, of resident cardiac progenitors derived from the SHF that specifically express Isl1 and have the potential to differentiate into cardiomyocytes, smooth muscle and endothelial cells. Through analysis of a transgene integration site position effect we identified Hes1, a member of the Notch signaling pathway, as a novel regulator of SHF development. Hes1 is expressed in the SHF during heart tube elongation. Hes1-/- hearts present outflow tract alignment defects (overriding aorta and ventricular septal defects). At earlier developmental stages, mutant embryos display SHF proliferation defects, elevated levels of the cell cycle inhibitor p27kip1, a reduction in cardiac neural crest cells and fail to completely extend the outflow tract. Given the importance of Isl1 as a marker of resident progenitor cells in the later heart we investigated whether other SHF regulators (Fgf10 and Hes1) contribute to myocardial progenitor cell fate in the fetal heart. Preliminary results reveal impaired proliferation of fetal cardiomyocytes in Fgf10-/- hearts while Isl1-positive cell numbers are increased. In contrast, Hes1 deletion impacts negatively on Isl1-positive cell numbers. Together, our results identify Hes1 as a novel regulator of SHF deployment and reveal that Fgf10 and Hes1 may regulate cardiac progenitor cell fate and cardiac growth during the fetal period.

95. Hematopoietic cell contribution to embryonic mouse valve development and adult valve homeostasis

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Heart valve interstitial cells are traditionally thought to originate from the embryonic endocardium through epithelial to mesenchymal transformation. Contribution of neural crest-derived and epicardially-derived cells has also been described. Here we report that cells of hematopoietic origin contribute to avian cushion/valve development. Using immunohistochemical analyses, we detected CD45+ (common leukocyte antigen) cells in chicken endocardial cushions starting from HH st 23-24. Their numbers peak at HH st 29 but CD45+ cells persist throughout embryonic development. These cells exhibit a specific atrial-side localization in the AV valves and ventricular-side localization in the semilunar valves. Quail-chick chimeric experiments, combined with QH1 immunolabeling, provided further support for the hematopoietic origin of embryonic valve cells. Next we investigated the contribution of hematopoietic-derived cells to mouse embryonic valve development. To accomplish this, we performed immunoluorescence analysis of CD45+ cells in mouse endocardial cushions from 9.5 dpc through post-natal life. The dynamics of CD45+ cell localization were similar to that observed in the avian system. We detected cells of hematopoietic origin in the cushions starting from 11.5 dpc and peaking by day 13.5 dpc. Moreover, the specific localization pattern observed in chicken valves was present in the mouse from embryonic stages through post-natal life. These findings support our previous reports where transplanted EGFP+ bone marrow cells were shown to contribute to the adult murine valve interstitial cell population in recipient mice. Collectively, our findings suggest that hematopoietic-derived cells contribute to valve development and homeostasis during both pre- and post-natal life and may contribute to valve pathologies.

ORAL SESSIONS
FRIDAY 21 MAY

Location: Great Hall

09.00 - 10.40 Epicardium
11.20 - 13.00 Cardiac Signaling
14.30 - 16.30 Genetics & Cardiovascular Malformations
17.00 - 18.45 3D-Techniques & Imaging

Epicardium

31. Epicardial fate and Notch signaling

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Lineage-tracing studies in chick demonstrated that epicardial cells give rise to endothelial and support cells of the coronary vasculature, and cardiac fibroblasts. Genetic fate mappings in the mouse have recently suggested that a substantial fraction of ventricular cardiomyocytes may also be of epicardial origin. This finding is relevant in the light of experiments that showed that the epicardium represents a cellular source for revascularization during cardiac regeneration in the zebrafish. It is therefore an exciting challenge to search for signaling pathways that mediate epicardial differentiation and to exploit their potential in the wounded adult mammalian heart. Given the controversy on epicardial cell fates in mammals, we reevaluated this problem by using a genetic lineage tracing system in the mouse. The Tbx18 gene is expressed in the left ventricle and ventricular septum, and the epicardium. Thus, a Cre Knock-in into the Tbx18 locus allows to trace the fate of epicardial cells overlying the right ventricle in mice double heterozygous for Tbx18Cre and a suitable reporter gene. Double immunofluorescence analysis of differentiation markers and a sensitive GFP reporter did not detect any right ventricular cardiomyocytes to be of epicardial origin. We will describe the cell types to which epicardial cells were found to contribute using this lineage system. Since Notch signaling has been implicated in vascular development, we wanted to explore the role of this pathway in epicardial differentiation and coronary vessel formation. We used both conditional Tbx18Cre mediated loss-of-function and gain-of function strategies. We will report on our findings that Notch signaling is required and sufficient for differentiation of certain cell lineages of the embryonic epicardium in the mouse.

32. Loss of the Type III Transforming Growth Factor-β Receptor decreases Hyaluronic Acid responsiveness in Epicardial Cells

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Mice lacking Tgfbr3 die at E14.5 due to failure of coronary vessel formation. Epicardial cells contribute to the formation of coronary vessels. Hyaluronic acid, a major extracellular matrix (ECM) component of the embryonic heart, has recently been reported to regulate epicardial cell signaling, specifically regulating invasion and differentiation. To determine if altered sensitivity to the ECM occurs after loss of Tgfbr3, we used immortalized epicardial cell lines from E11.5 embryos to determine the invasive potential of epicardial cells in response to high molecular weight hyaluronic acid (HMW-HA) using a modified Boyden chamber assay. Tgfbr3+/+ cells demonstrate a dose dependent response to HMW-HA from 0-300 µg/ml, whileTgfbr3-/- cells do not. Incubation of Tgfbr3+/+ cells with 300 µg/ml HMW-HA induced a 3.78-fold increase in invasion, relative to vehicle, into a collagen 1 matrix. Tgfbr3+/- and Tgfbr3-/- cells showed a 2.39 and 2.30-fold increase in invasion, respectively. Since growth factors like TGFβ can induce transformation and differentiation, we next asked if HMW-HA could induce differentiation. In Tgfbr3+/+ cells, HMW-HA induces a 40-fold increase in Smooth Muscle α-actin (SmαA) expression, but no change in the smooth muscle markers, Smooth Muscle 22α (SM22α) or calponin. In Tgfbr3-/- cells, SMA increased only 6.8 fold. HMW-HA does not induce the endothelial markers Tie2, Flk1, or VE-cadherin in either genotype. These data demonstrate that Tgfbr3 is required for the maintenance of epicardial cell responsiveness to hyaluronic acid. Loss of responsiveness to this ECM component may contribute to phenotype seen in Tgfbr3-/- mice.

33. NFATc1 in epicardium-derived cells is required for fibrous skeleton and intramyocardial coronary vessel development

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The proepicardial organ (PE) contributes cells to the epicardium, coronary vasculature, and fibrous skeleton. During heart looping, PE cells migrate over the myocardium to form the epicardium. A subset of epicardial cells, epicardium-derived cells (EPDCs), undergo EMT, migrate into the subepicardial space and invade the myocardium. NFATc1 is a transcription factor integral to the transition from endocardial cushion growth to valve leaflet remodeling. NFATc1 also is expressed in mouse and chick PE, epicardium and EPDCs. We hypothesize that NFATc1 promotes epicardial cell proliferation and EPDC invasion via ECM remodeling enzyme expression. NFATc1-/- mice have decreased epicardial cell proliferation at E10.5. WT1-Cre mice were bred with NFATc1-flox mice to determine the role of NFATc1 in EPDC development. Epicardial loss of NFATc1 leads to loss of fibrous skeleton and intramyocardial coronary vessels at E17.5 as well as perinatal lethality. Loss of NFATc1 in EPDCs also decreases Cathepsin K (CtsK), an enzyme associated with myocardial invasion. In Chick PE cell cultures RANKL treatment increases expression of CtsK via a Calcineurin-dependent mechanism, as in remodeling valves. These data are consistent with NFATc1 regulation of EPDC invasion required for coronary vessel and fibrous skeleton development. Overall, these analyses demonstrate a critical role for NFATc1 in epicardial cell proliferation and ECM remodeling analogous to that observed in the developing valves.

34. PDGF Signaling is Required for Epicardial Functions and New Blood Vessel Formation in Regenerating Zebrafish Hearts

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A zebrafish heart can fully regenerate after amputation of up to 20% of its ventricle. During this process, new coronary blood vessels form to revascularize the regenerating tissue. The formation of new coronary blood vessels during zebrafish heart regeneration likely recapitulates embryonic coronary vessel development, which involves the activation and proliferation of the epicardium followed by an epithelial-to-mesenchymal transition (EMT). The molecular and cellular mechanisms underlying these processes are not well understood. Here, we examine the role of platelet-derived growth factor (PDGF) signaling in explant-derived primary cultured epicardial cells in vitro and in regenerating zebrafish hearts in vivo. We observe that EMT and mural cell markers are upregulated in the regenerating hearts. The epicardium of 7 dpa (days post amputation) and 4 dpa regenerating heart explants undergoes EMT and proliferation, respectively, and PDGF signaling is both necessary and sufficient for these processes in vitro. PDGF-induced EMT is mediated by Rho associated protein kinase (ROCK), whereas epicardial cell proliferation is mediated by PI3 kinase. Inhibition of PDGF signaling in vivo blocks epicardial cell proliferation, EMT, and blood vessel formation. Our data suggest that PDGF signaling plays important roles in epicardial cells that contribute to heart regeneration in zebrafish.

35. PDGFR signaling in the epicardium is required for epicardial EMT and coronary VSMC and cardiac fibroblast development

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Coronary vascular smooth muscle cells (cVSMC) and cardiac fibroblasts are epicardial derived cell (EPDC) populations essential for normal coronary artery development and are important mediators of myocardial pathogenesis. These cells form when a subset of epicardial cells undergoes an epithelial-to-mesenchymal transition (EMT) and migrate into the myocardium. The two PDGF receptors (PDGFRα and PDGFRβ) are both expressed in the embryonic epicardium and in EPDC. To examine their function during heart development we generated mice with epicardial specific deletions of the PDGF receptors. Interestingly, each receptor was required for development of a unique epicardial derivative, PDGFRβ for cVSMC and PDGFRα for cardiac fibroblasts. When both PDGF receptors were deleted, we observed a complete loss of EPDC. Ex vivo tracing of epicardial cells confirmed a failure of migration. PDGF receptor mutant hearts also exhibited decreased vimentin expression in the epicardium, suggesting an EMT defect. We determined that transcriptional markers of EMT (Snai1, Snai2, Sox9) were reduced in E12.5 hearts and primary epicardial cultures. TGFβ and PDGF stimulation of mutant epicardial cultures also showed defective EMT activation. Our current data suggests Sox9 is induced by PDGF signaling and that expression of Sox9 partially rescues the mutant EMT phenotype. Additionally, PDGF stimulation leads to phosphorylation of Sox9 and combined with TGFβ, induces a transcriptional complex with Smad2/3 and Snai1. These data establish PDGF signaling through Sox9 as a novel pathway essential for epicardial EMT and demonstrate a unique requirement for each receptor in the formation of cVSMC and cardiac fibroblasts.

Cardiac Signalling

1. Inhibition of Bmp signaling by Smad6 is required for terminal differentiation of ventricular cardiomyocytes.

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Using zebrafish as a vertebrate model to study mechanisms that regulate growth of the embryonic heart tube we previously identified two distinct phase when new cardiomyocytes are added to the growing linear heart tube; first a continuous phase of cardiomyocyte addition starts in the ventricle and ends in the atrium followed by a later phase when cells are added to the arterial pole. Although we identified Isl1 and Fgf signaling to be essential for specific steps during these processes we had not yet identified the signal that regulates the continuous recruitment of new cardiomyocytes. Here we show, using novel and existing zebrafish mutants and transgenic lines, that Bmp signaling is essential for the continuous recruitment process and that this requirement for Bmp signaling is independent from its role in dorsoventral patterning. Analysis of Bmp signaling in the lateral plate mesoderm during cardiomyocyte differentiation revealed heightened signaling prior to differentiation, followed by a rapid down-regulation of Bmp signaling in differentiating ventricular myocytes. Using a loss-of-function approach we have demonstrated that Smad6 is responsible for mediating the down-regulation of Bmp signaling in differentiating myocytes. In addition, forced Bmp activation in differentiating myocytes prevents terminal differentiation and results in hearts with smaller ventricles. In conclusion our data demonstrates a biphasic role for Bmp signaling during elongation of the linear heart tube by first inducing cardiomyocyte specification followed by an active repression of BMP signaling to allow their terminal differentiation.

2. The BMP Pathway Acts to Regulate Tbx20 in the Developing Heart

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TBX20 has been shown to be essential for vertebrate heart development. Mutations within the Tbx20 coding region are associated with human congenital heart disease, and the loss of Tbx20 in a wide variety of model systems leads to cardiac defects and eventually heart failure. Despite the critical role of TBX20 in a range of cardiac cellular processes, the signal transduction pathways that act upstream of Tbx20 remain unknown. Here we have identified and characterized a conserved 334bp Tbx20 cardiac regulatory element and shown that this element is directly activated by the BMP/SMAD1 signaling pathway. We demonstrate that this element is both necessary and sufficient to drive cardiac-specific expression of Tbx20 in Xenopus, as well as in the mouse, and that blocking SMAD1 signaling in vivo specifically abolishes Tbx20 transcription in the developing heart while having no effect on other cardiac markers including Tbx5, myosin heavy chain, and tropomyosin. We further demonstrate that activation of Tbx20 by SMAD1 is mediated by both canonical and non-canonical SMAD-binding sites in vitro and have gone on to show that phospho-SMAD1 directly binds sites within this element in vivo. Collectively, our findings define Tbx20 as a direct transcriptional target of the BMP/SMAD1 signaling pathway during cardiac maturation.

3. Bmp-signaling regulates myocardial differentiation from cardiac progenitors through a micro RNA-mediated mechanism

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MicroRNA's (miRNA) are small, non-coding RNA's that regulate gene expression post-transcriptionally. Because the signals that regulate miRNA expression remain poorly understood, we investigated Bone Morphogenetic Protein (Bmp)-signaling in miRNA regulation in Isl1-lineage cardiac progenitors. Our findings indicate that Bmp2 and Bmp4 regulate OFT myocardial differentiation via regulation of the miRNA 17-92 cluster. In Bmp mutant embryos, expression of multiple miRNAs encoded by the miRNA 17-92 cluster was reduced. Importantly, we uncovered functional miRNA 17 family seed sequences within the 3' UTR of the cardiac progenitor gene Isl1. In Bmp mutants, Isl1 expression failed to be silenced and myocardial differentiation was disrupted. Transfection experiments indicated that miRNA 17 and miRNA 20a repressed Isl1 expression. Moreover, genetic interaction studies uncovered a synergistic interaction between miRNA 17-92 cluster and Bmp4 providing direct in vivo evidence for the Bmp-miRNA 17-92 regulatory pathway. Together, our findings indicate a novel, miRNA-mediated effector mechanism for Bmp-regulated cardiac progenitor differentiation.

4. Cardiac Laterality is Controlled by Multiple Rho Kinase Signaling Pathways in Ciliated Cells

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Asymmetric fluid flow generated by embryonic ciliated cells conveys laterality signals to the developing heart. In zebrafish, asymmetric flow is produced by a ciliated epithelium in Kupffer's vesicle (KV), which provides a useful model for identifying pathways regulating the ciliated cells that control cardiac laterality. Taking a candidate approach, we analyzed the role of Rho kinase signaling in KV. Rho kinase (Rock) proteins are effectors of Rho GTPases that control several cell behaviors and can mediate planar cell polarity (PCP) signals. Interfering with Rho kinase signaling via dominant negative Rock constructs or antisense morpholino (MO) knockdown of the zebrafish Rock homologs rock2a or rock2b altered asymmetric Nodal signaling and randomized heart laterality. Videomicroscopy of beads injected into KV demonstrated that asymmetric flow was disrupted in rock2a and rock2b morphants. Cilia were truncated in rock2a morphants, consistent with a role for PCP signaling during KV ciliogenesis. However, cilium length was unaffected in rock2b morphants. Analysis of the cellular architecture of KV showed that the arrangement of cells along the anterior-posterior (AP) axis was disrupted by rock2b knockdown. In wild-type embryos >60% of ciliated cells were tightly packed into the anterior region of KV, the site of strong asymmetric flow. This anterior-biased distribution was lost in rock2b morphants. Time-lapse imaging of embryos expressing GFP in KV cells revealed that rock2b regulates cell morphologies that appear to mediate KV architecture. Our results uncover a Rho kinase signaling pathway that controls ciliogenesis and a second pathway involved in establishing AP asymmetry in KV.

5. Mitochondrial structure and function during cardiac myocyte differentiation

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Although oxidative metabolism provides energy in the mature cardiac myocyte, the early embryo is thought rely mainly on anaerobic glycolysis and not aerobic, mitochondrial respiration. However, little is known about cardiac energetics and mitochondrial function in the embryo, and we hypothesize that changes in mitochondrial structure and function during embryonic cardiac development are critical for normal myocyte differentiation and cardiac morphogenesis. To test this hypothesis, we measured respiration and examined mitochondrial structure and function in whole embryonic hearts and cultured myocytes using light and electron microscopy. Mitochondria of embryonic day (E) 9.5 ventricular myocytes had an electron-lucent matrix and immature inner mitochondrial membrane (IMM) architecture, were shorter in length and less branched, and did not associate closely with the contractile apparatus but resided primarily around the nucleus and cell periphery. By E13.5, mitochondria had a dense matrix and mature IMM ultrastructure and were longer, branched, networked, and more closely associated with the contractile apparatus. Functional measurements demonstrated dramatic increases in mitochondrial membrane potential, calcium uptake, and electron transport chain activity and decreases in oxidative stress as the heart developed. These structural and functional and data suggested an increase in IMM permeability, and closure of the mitochondrial permeability transition pore using cyclosporin A or cyclophilin-D null embryos caused premature maturation of mitochondrial structure and function. Taken together, these data suggest that maturation of mitochondrial structure and function may regulate cardiac myocyte differentiation.

ESC-WG DAP session Genetics & Cardiovascular Malformations

204. Dysregulation of the PDGFRA gene causes inflow tract anomalies including TAPVR: Integrating evidence from human genetics and model organisms

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Congenital heart defects (CHD) usually occur sporadically via interplay between genetic and/or environmental factors. For most CHD, pinpointing these factors has proven problematic. We used a combined approach in humans and model organisms to identify a susceptibility factor for one such CHD, total anomalous pulmonary venous return (TAPVR). We report genetic analyses in TAPVR patients that implicate the PDGFRA gene in the development of TAPVR. Gene expression studies in mouse and chick embryos for both the Pdgfra receptor and its ligand Pdgf-a show temporal and spatial patterns consistent with a role in pulmonary vein (PV) development. Functional knockdown of PDGF-signaling in both chick and mouse during the period of PV formation causes a spectrum of inflow tract defects, including TAPVR, that involve the dorsal mesocaridal protrusion (DMP) supporting a role for PDGF-signaling in second heart field (SHF) development. These defects occur with low penetrance (~7% for TAPVR and ~30% for intermediate anomalies) suggesting the interaction of other genetic or environmental factors. Failure of a specific human PDGFRA BAC transgene to rescue inflow tract defects of Pdgfranull/null embryos further supports dysregulation of PDGFRA in human TAPVR. We also show that the TAPVR seen in chick and mouse is highly similar to that discovered in an abnormal early stage embryo from the Kyoto human embryo collection. Taken together, these data from human genetics and animal models support a role for PDGF-signaling in normal PV development and provide important insight into the embryogenesis and molecular pathogenesis of TAPVR, a CHD with complex inheritance.

205. Effects of conditional deletion of hypoxia-inducible factor (hif) and hypoxic stress on mouse heart morphogenesis

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Oxygen gradients have been identified in mid-gestation avian and mouse heart tissues, with the OFT, AVJ and IVS myocardium and cushion mesenchyme relatively hypoxic. We hypothesize that: 1) hypoxia/HIF is required for the remodeling of these tissues in the transition to the mature four chambered heart. This is supported by the lethality of germ line inactivation of HIF-1 at ~E11.5, while its cell-specific function has not been identified 2) these tissues in this developmental window are most susceptible to hypoxic stress. This is supported by epidemiologic studies showing increased prevalence of CHDs in Tibetans living at very high altitudes. We conditionally inactivated HIF-1a in the mouse with Cre drivers (cKO) and tested for gene-environment interactions with hypobaric hypoxic stress (0.5 ATM) in the proposed window of vulnerability (E10-13). HIF-1a cKO in neural crest (Wnt1Cre) caused postnatal lethality with high penetrance; the few surviving mice were severely runted without major CHDs. OFT defects were observed in E16.5 mice with low penetrance. HIF-1a cKO in second heart field myocardium (MEF2cCre) also resulted in low penetrance of OFT defects. HIF-1a flox mice were crossed with Tamoxifen-inducible b-actin Cre and treated with Tamoxifen at E10 for temporally regulated HIF-1 inactivation; this data will be presented. Mice exposed to hypobaric hypoxia (0.5 ATM) from E10.5-13.5 showed a high incidence of VSD and other outlet defects at E15.5 with no apparent genotype-phenotype interactions. We propose that hypoxia/HIF plays a critical role in morphogenesis of outlet structures of the mid-gestation mouse heart, and that this process is susceptible to hypoxic stress. Gene or cell compensations may moderate the effects of hypoxic stress or HIF-1a inactivation.

206. p53-related p63 transcription factor is essential for cardiac morphogenesis

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The transcription factor p63, a member of the p53 family, is essential for skin morphogenesis and epithelial stem cell maintenance. Indeed, p63-deficient mice lack epidermis and feature many defects in epithelial development. Here, we report an unexpected role of p63 in heart development. p63 null mice exhibit severe defects in embryonic cardiac development, including pronounced dilation of both ventricles, a defect in trabeculation and abnormal septation. This was accompanied by myofibrillar disarray, mitochondrial disorganization and reduction in spontaneous calcium spikes. The use of embryonic stem (ES) cells, that recapitulate in vitro the main steps of embryogenesis, shows that p63 deficiency prevents expression of pivotal cardiac genes and in turn cardiogenesis, resulting in the absence of beating cardiomyocytes. Coculture of p63 knock-down ES cells with wild type ES cells, supplementation with Activin A or overexpression of GATA-6 rescue cardiac gene expression and beating activity of ES cell derived cardiomyocytes. These observations indicate that p63 acts in a non-cell-autonomous manner by modulating expression of endodermal factors. Accordingly, p63 is expressed by sox-17-positive endodermal cells and binds to the Activin A regulatory sequences in vivo. Our findings uncover a critical role for p63 in cardiovascular function and indicate that congenital heart defects could be an underdiagnozed feature of human p63-related syndromes.

207. Copy Number variations (CNV) in left sided congenital heart disease

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Purpose: Left sided congenital heart disease encompass a spectrum ranging from bicuspid aortic valve to hypoplastic left heart syndrome. We hypothesized that rare de novo and rare familial Copy Number variations (CNV) explain a considerable amount of the complex genetic traits seen in its pathogenesis. Methods: We genotyped 396 individuals in 44 families at high resolution (Affymetrix 6.0 N affected = 193, N unaffected = 203). Unaffected individuals from the same cohort and a cohort with myocardial infarction (N=862), but no valve disease were used as controls. Results: Our analysis revealed 33 rare CNVs. 30 have been only detected in a single individual or family. 3 have been identified in independent families. Public databases and gene chip data show cardiac and/or endothelial expression for genes adjacent to or overlapping with the identified CNVs. Among the genes identified several showed a GTPase regulator activity and a role in chromosomal maintenance. Preliminary functional experiments in Xenopus laevis support a distinct role for the identified genes in the cardiovascular development. Conclusions: The high prevalence of private CNVs is suggestive of genetically heterogeneous determinants contributing to congenital left sided heart defects. Functional data and in silico analysis of the genes identified point to a role in cardiac pathogenesis. Our approach holds great promise for the further genetic dissection of congenital heart disease especially aortic valve disease as a complex trait caused by rare alleles with major effects.

208. The role of FKbp12-notch1 mediated regulation in ventricular trabeculation and compaction

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Ventricular trabeculation and 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 is largely unknown. One of the best known mouse models is the FK506 binding protein 12 (Fkbp1) mutant mice, which mimics the NLVM phenotype. It has been an assumption that hypertrabeculation and noncompaction are cardiomyocyte-autonomous defect. However, our initial attempt to rescue Fkbp1-deficient mice using cardiomyocyte-restricted Fkbp1 transgenic mice was failed, indicating that cardiomyocyte may not be the only cause of cardiac hypertrabeculation and noncompaction in Fkbp1-deficient mice. Therefore, we went on to have generated a series of Fkbp1 conditional knockouts, including cardiomyocyte specific (Fkbp1ckomc), endothelial specific (Fkbp1ckoen), neural crest cell specific (Fkbp1ckonc), and epicardial specific (Fkbp1ckoep). While all these different strains of mutant mice have normal cardiovascular phenotypes, only endothelial specific Fkbp1ckoen mice embryo produced ventricular hypertrabeculation and noncompaction. Further analysis of potential molecular mechanism for this striking phenotype revealed that Fkbp12 is involved in regulating NICD1 stability. NICD protein level was significantly upregulated in Fkbp1ckoen endocardial endothelium. This is further confirmed by Hes1-luciferase assay. In contrast, overexpression of Fkbp12 significantly shortens NICD1 half-life. Taken together, our finding suggests that the endocardial endothelium is critical in controlling the ventricular trabeculation and/or compaction and that Fkbp12-Notch mediated signaling pathway plays an important role in this intercellular communication.

209. MCTP2 is a novel gene causing aortic coarctation

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Coarctation of the aorta (CoA) accounts for one tenth of all congenital heart anomalies. Despite the frequency, the causative genes underlying this relatively common left ventricular outflow tract (LVOT) defect remain largely unknown. We identified two half-siblings with CoA with a 2.2 Mb genomic deletion involving 15q26.2, inherited from their mother, who was mosaic for this deletion. This interval contains a single evolutionary conserved gene, MCTP2 (multiple C2-domains with two transmembrane regions 2). Using MCTP2-targeted array CGH screening, another individual with non- syndromic CoA and hypoplastic left heart was found to have a de novo intragenic tandem duplication within MCTP2, predicted to result in premature truncation of the protein, p.F697X. We then sequenced the MCTP2 gene in 142 patients with LVOTO and found five missense variants not found in 200 ethnically matched controls. In vitro functional analysis of two of these missense variants within the C2A domain of the protein showed altered Ca2+ binding affinity. To further investigate the role of MCTP2 in cardiac development, we carried out loss of function experiments by injecting Xenopus laevis embryos with a splice blocking antisense morpholino oligonucleotide. Morphant embryos showed failure of endocardial cushion formation throughout the developing common outflow tract, confirming the functional importance of this gene in cardiogenesis. Our results identify MCTP2 as a novel genetic cause of CoA and related cardiac malformations.

Netherlands Heart Foundation session 3D-Techniques and Imaging

169. 3D Modelling of heart development: looking to the future

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The complexity of heart morphogenesis remains peculiarly challenging, whether the goal is to better understand normal development or to establish the origins of cardiac malformations. However until relatively recently, 3D modelling of the developing heart has been largely restricted to illustrative purposes rather than a significant research tool. The past few years have seen important changes, driven by the dramatic increase in easily accessible computing power and the development of novel or newly adapted 3D imaging techniques. As a result, it is now realistic for 3D analysis to be adopted as an investigative tool that can be allied to more conventional molecular and cellular analysis of cardiac development.
However, it is also clear that, for the moment, the experience and expertise in such modelling remains confined to a small number of laboratories. As the first Weinstein meeting to schedule a specific session on 3D analysis and to help in its adoption by a wider range of labs, it is timely to try and suggest some of the key lessons and challenges of this work. Integrating 3D analysis (whether of gene expression, morphological development or embryonic heart function) into existing research also raises particular problems of data handling, including common approaches for data analysis and methods for simplifying data presentation or publication. Central to both is the sheer quantity of data generated by 3D analysis and the opportunities this presents if appropriate ways can be found to share this information.

170. Imaging in the Embryonic Heart: morphodynamic studies with Optical Coherence Tomography (OCT)

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In vivo analysis of cardiac physiology and non-invasive imaging of the beating early embryonic heart in 2D and 3D remain a challenge in cardiovascular development research. Further, high-resolution 3D and 4D in vivo imaging of early phases of cardiovascular development over long periods of time under constant physiological conditions (e.g. temperature, humidity) is another challenge in these studies. Without appropriate maintenance of temperature, for example, the embryonic heart rate declines rapidly and often results in an increase in regurgitant flow. However, preload, afterload, and the effects of blood flow on cardiac morphodynamics are critical parameters that have to be examined more closely for a better understanding of normal and abnormal cardiovascular development. I will give in this talk a short overview on optical coherence tomography (OCT) as a non-invasive high-resolution imaging tool for studies in cardiovascular development. I will share examples of data to demonstrate the first realization of an OCT system integrated into a new environmental incubation chamber to facilitate real-time in vivo imaging of cardiovascular development in chick embryos under stable physiological conditions during examination of the embryos. Because uncovering of the pumping mechanism of tubular embryonic hearts requires detailed information about the hemodynamics as well as morphological dynamics of their pump action, I'll also show some results from our studies related to this end for early stages of cardiac development using OCT and ist component M-mode OCT. Finally, I'll share the first 4D-OCT movies of the beating early embryonic heart in chick embryos acquired with a novel technique that uses a rotational scan mode of a swept-source OCT system (Thorlabs) using retrospective gating.

171. How to quantify in 3D: imaging of gene expression patterns and morphogenetic parameters in the embryonic heart.

Jan M. Ruijter, Alexandre T. Soufan, Jaco Hagoort, Bouke A. de Boer and Antoon F.M. Moorman
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Gene expression profiling projects have provided us with increasing knowledge about overall gene activity levels in developing, healthy, and diseased tissue. For the functional interpretation of this wealth of information, the need to know in which part of the organ or tissue these genes are expressed becomes more and more pressing. Specific histological staining of proteins (with ICC) or mRNAs (with ISH) is therefore used to visualize these gene products, and thus the level of gene expression, in sections. Similarly, morphogenetic processes, like cell proliferation and apoptosis, can be visualized with histological procedures. In structurally complex organs the interpretation of the resulting sections is hampered by the loss of 3D morphology. The required level of detail and the limited penetration of staining agents in whole mount staining procedures dictate the use of serially sectioned biological material and make the use of 3D computer reconstructions unavoidable.
It is also our contention that ongoing discussions in embryology and anatomy will only be resolved by making 3D reconstructions of the dynamic and complex morphology of the (developing) heart, associated with the patterns of relevant genes. The 3D reconstruction protocol can be broken down into parallel qualitative (i.e. morphology) and quantitative (i.e. cell proliferation) methods. The qualitative method identifies the organ or tissue of interest (i.e. cardiac muscle cells), resulting in a surface reconstruction. The quantitative method relies on a specific staining method to identify individual (i.e. proliferating) nuclei. The number of nuclei is then systematically measured, providing local 3D information on i.e cell proliferation rate. Mapping of these local data onto the morphological surface reconstruction results in a reconstruction that not only conveys morphological information, but also quantitative morphogenetic data. Similarly local measurement can be applied on the staining intensity resulting from ICC and ISH staining and can thus be used to quantitatively reconstruct gene expression information in association with morphogenetic parameters.

172. How to place a section: Automated fitting of histological sections into a high resolution 3D reference model of a developing mouse heart

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The rapid morphological change of the developing heart makes it difficult to position a single section within a heart. This seriously hampers the interpretation of the section in its spatial context. To retrieve the postion of a single section through a heart, we developed a program (TRACTS) which fits arbitrary histological sections into a high resolution episcopic 3D heart model. We implemented a straightforward brute force pixel-based approach, where the (2D) input section is compared to a selection of virtual (2D) cross sections of the (3D) reference model. Model cross sections are selected on basis of four image features: size, overall density, regional density and center of mass. We describe how the performance of the program was tested. The performance is assessed using virtual cross-sections in arbitrary directions from a second, episcopic, dataset. To check whether the results are valid for paraffin embedded material, the program was also tested using paraffin sections taken from reconstructed in situ hybridization datasets. A third and last test involved a panel of morphological experts, who manually fitted a subsample of the episcopic input set to the reference model. The program outperformed the individual experts and, in about 30 seconds per section, fits just as well as the best fits found by five morphological experts. The results show that the program is quite robust for differences in developmental stage. The use of the program will help proper annotation of structures in sections and therefore improve interpretation and communication on experimental results.

173. How to make a 3D pdf: From 3D-reconstruction to interactive 3D-pdf; a protocol for biologists.

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Anatomical and embryological studies heavily rely on three-dimensional (3D) data sets, which allow the proper understanding of complex morphogenetic processes and changing patterns of gene expression. These 3D data lead to new insights, which may be accurately discussed within the research group because the right tools (i.e. proper software) are on hand. However, dissemination of such data is severely hampered due to the inherently 2D paper medium used for publication. This is very unfortunate, as many insights are very hard to convey without interaction with the 3D data. An opportunity to remedy this has presented itself, as Adobe Acrobat Pro Extended allows to embed 3D objects into a pdf document. The read-only freeware version Adobe Reader is universally used and virtually all scientific papers are distributed in this format. Using Adobe Reader to interact with 3D objects is reasonably straightforward; creating such objects, however, is not. Therefore, we developed a protocol that describes how one can embed a 3D object into a pdf file and enhance it with more functionality. We will show -step by step- how to create a basic 3D-pdf which is useful for quick exchange of scientific findings. Some more advanced additions will be applied, resulting in a 3D-pdf that is ideally suited for presentation or publication. Enriching documents with 3D objects is perfect in the sense that it is universal; every reader can interact with the 3D content. Furthermore, it enables researchers to better convey the ideas resulting from their 3D analyses, whereby miscommunication is reduced.

ORAL SESSIONS
SATURDAY 22 MAY

Location: Great Hall

09.00 - 11.00 Valve Development
11.45 - 12.25 Heart Fields
12.25 - 13.05 Keynote Lecture II
14.30 - 16.10 Neural Crest & Conduction System
16.40 - 17.40 Cardiomechanics
17.40 - 17.50 Weinstein Conference 2011

Valve Development

69. MiR-23 is essential to restrict endothelial-to-mesenchymal transition during cardiac valve formation.

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Endothelial-to-mesenchymal transition (endo-MT) in the endocardial cushions (ECs) is a critical step during valvulogenesis. Although several factors have been identified that induce and/or are required for endo-MT, very little is know about mechanisms that restrict endo-MT to the ECs located in the AV canal. MicroRNAs (miRs) are small non-coding RNAs and we found that miRs of the miR-23,24,27 clusters are highly expressed in ECs of the zebrafish embryo. Analysis of dicer mutants, which lack all mature miRs, revealed unrestricted endo-MT occurring in the entire endocardium of the chambers. Knock-down of individual miRs showed that miR-23 is both necessary and sufficient to restrict the number of endothelial cells in the endocardium that transdifferentiate and contribute to valvulogenesis. Furthermore, our results demonstrate that miR-23 is required to dampen and eventually stop the transdifferentiation response in the ECs. Using an in vitro approach we show that miR-23 can target hyaluronan synthase 2 (has2), an enzyme producing extracellular hyaluronan, which is essential for endo-MT. In addition our genetic rescue experiments demonstrate that the upregulation of Has2 activity seen in embryos lacking miR-23 is responsible for the enhanced and ectopic endo-MT. Since miR-23 is highly expressed in ECs after endo-MT has been initiated we propose a model in which induction of miR-23 expression in ECs provides a negative feedback mechanism to restrict and dampen the endo-MT process allowing maturation of the valve leaflets.

70. Cardiac valve malformations: new insights from Pdlim7, an unexpected suspect in heart development

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PDZ-LIM proteins contain multiple binding domains, facilitating interactions with the actin cytoskeleton, nuclear factors, and signaling molecules, thereby allowing the proteins to carry out diverse biological functions. Here, we characterize a new family member, Pdlim7 that appears to have important functions during cardiac development, as evidenced by specific malformations resulting from inactivation in zebrafish and mouse models. Knock-down of Pdlim7 in the zebrafish causes misregulation of genes in the atrioventricular (AV) canal leading to heart looping defects and valve malformations. Extending these studies to the mouse, we generated a Pdlim7 knock-out allele using a gene-trap approach with the insertion of lacZ between exons 2 and 3. Pdlim7 is expressed in a spatial and temporal manner in the developing mouse heart and its activity is extended into adulthood. Pdlim7 null embryos reveal misregulation of AV canal genes and increased AV cushion tissue during early valvulogenesis, resulting in impaired maturation leading to hypertrophic adult AV valves. 3-dimensional reconstructions from adult heart serial sections demonstrate these morphological changes following loss of Pdlim7. Supporting the structural problems in adult mice, echocardiography studies of Pdlim7 mutant mice reveal increased tricuspid and mitral annulus dimensions with decreased left ventricular (LV) diastolic function. The cardiac problems observed in adult mice lacking Pdlim7 proteins appear to result from early problems in cardiogenesis that manifest themselves postnatally. Thus, these findings establish Pdlim7 as an important player in heart development and suggest that the Pdlim7 knock-out mouse may provide a new model for cardiac valve disease progression.

71. Krox20 is required during valve remodeling and maturation

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Heart valve replacement is the second most common cardiac surgery in the industrialized countries, and the majority of replaced aortic valves have congenital malformations. Two processes can affect the aortic valve – aortic stenosis and aortic insufficiency (also called aortic regurgitation). Despite high prevalence of these diseases the causes of valve developmental anomalies and associated dysfunction have not been identified. Using genetically modified mouse models, we found that the transcription factor Krox20 (also called Egr2) is expressed in leaflets of the cardiac valves, and its expression is maintained after birth. Krox20 inactivation results in lethality at birth due to a respiratory defect. No role of Krox20 in the heart development has yet been identified. Using high temporal and spatial resolution cine-MRI we examined the cardiac function of Krox20+/- adult mice and found that 60% of them have cardiac defects, including bicuspid aortic valve (BAV) and aortic regurgitation. Interestingly, Krox20-deficient embryos (E12.5) and fetuses (E17.5) exhibit reduction of mesenchymal cell proliferation and dysregulation of interstitial cell differentiation in the cardiac valves respectively. Conditional inactivation of Krox20 in endothelial-derived cells with Tie2Cre results in high percentage incidence of aortic regurgitation. Krox20 has been shown to be a target of the NRG1/ErbB signaling pathway in Schwann cells. We are employing the explants culture system to determine if the epithelial mesenchymal transition (EMT) is affected in Krox20-deficient hearts and if the NRG/ErbB signaling during this process regulates Krox20. This study reveals an important role of Krox20 in heart valves remodeling and maturation.

72. The Metalloprotease ADAMTS5 Plays A Critical Role In ECM Stratification And Cusp Sculpting During Semilunar Valve Maturation

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The extracellular matrix (ECM) undergoes dramatic remodeling during valve maturation, resulting in formation of the specialized ECM layers required to regulate blood flow in the adult heart. Since the loss of ECM stratification and accumulation of proteoglycans is a hallmark of valve disease, uncovering processes that regulate normal valve maturation is clinically significant. Here we investigate the role of the ECM protease ADAMTS5 (A Disintegrin-like And Metalloprotease domain with ThromboSpondin-type 1 motifs) that is expressed by the endocardium and a subset of mesenchymal cells in the hinge region of developing semilunar valves. A known substrate of ADAMTS5 in the developing heart, the ECM proteoglycan versican, is highly expressed and critical for early valve formation, but has limited and restricted expression in adult valves. We report that ADAMTS5 deficient mice develop severely enlarged and malformed semilunar valves with dramatically decreased versican processing reflected by accumulation of intact versican. Reduced mesenchymal cell compaction and increased cell proliferation further contribute to the enlarged semilunar valves in ADAMTS5 deficient mice. The “balloon-like” shape of the developing mutant cusps may also result from poor organization of fibrous ECM in ADAMTS5 nulls compared to wild type littermates. Increased nuclear phospho-Smad2 immunoreactivity strongly suggests that TGFβ signaling may be a consequence of Adamts5 deficiency, and contribute to abnormal valve development in ADAMTS5 deficient embryos. These studies identify a novel role for ADAMTS5 and versican cleavage in sculpting the cusps and stratifying the ECM during fetal valve maturation and identify a novel candidate gene for congenital valve defects.

73. Induction of a developmental gene program in pediatric and adult aortic valve disease

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Congenital heart valve malformations, including latent defects such as bicuspid aortic valve, are estimated to affect 1-2% of the United States population. There is increasing evidence that aortic valve (AoV) disease has a basis in development, and congenital valve malformations are prevalent in patients undergoing valve replacement surgery. The progression of AoV disease includes: activation of valvular interstitial cells (VICs), valve sclerosis due to abnormal ECM production/remodeling, followed by calcification of sclerotic lesions. Previous studies have defined a shared gene program between valve and bone progenitor cells. We hypothesize that reactivation of these genes during AoV disease constitutes an osteogenic-like process in VICs, leading to valve calcification. Explanted human pediatric and adult diseased AoVs were obtained following valve replacement surgery. Preliminary studies revealed increased expression of the proliferation marker phospho-histone H3 and alpha smooth muscle actin, indicating activated VICs, in areas of diseased valves displaying ECM disorganization. Pre-calcified regions of diseased adult AoVs have increased expression of Twist1, Mef2c, Msx2, Sox9, and phospho-SMAD 1/5/8, factors critical for valve progenitor and pre-osteoblast development. Calcified regions of diseased adult AoVs exhibit Runx2 expression, a marker of osteogenic bone formation. In pediatric valves, expression of both Mef2c and Twist1 is increased, however calcification and late osteogenic gene markers are not observed. These results demonstrate the expression of early markers of bone and heart valve development in diseased valves, supporting the hypothesis that a common developmental gene program related to embryonic valve progenitors and osteogenesis is reactivated during human pediatric and adult AoV disease.

74. Reduced Sox9 function promotes heart valve calcification in vivo

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Heart valve calcification is the third leading cause of adult heart disease, however, the mechanisms of onset and progression are unclear. Normal heart valves share molecular phenotypes with cartilage tissue, while calcified valves express osteogenic genes, suggesting similarities with bone development. During skeletogenesis, Sox9 promotes cartilage formation by positively promoting expression of cartilage-associated genes. In persistent cartilage, Sox9 inhibits activity of the osteoblast transcription factor RUNX2, therefore repressing activation of osteogenic target genes. The purpose of this study was to test the hypothesis that in heart valves, Sox9 is required to maintain extracellular matrix homeostasis by promoting cartilaginous phenotypes and preventing osteogenic processes. Heart valves from mice with reduced Sox9 function in a subset of valve cells express decreased levels of cartilage-associated proteins, and develop calcific lesions on the surface of mitral, tricuspid and aortic valve leaflets. This calcification is associated with increased expression of osteogenic genes including runx2, osteopontin, and osteonectin at 12 months, similar to findings in human calcified valves. To determine the direct downstream targets of Sox9 that mediate this osteogenic phenotype in heart valves, we have employed combinatory ChIP-on-ChIP and microarray approaches, along with Sox9 knockdown and overexpression studies in heart valves and cartilage tissue. Using these approaches we have identified novel putative targets of sox9 regulation osteopontin (spp1), cingulin (cgn), and arginase (arg1). Ongoing studies will further define the roles of these candidate genes in heart valve calcification. Collectively these studies have identified reduced Sox9 function as a developmental origin for adult calcific valve disease.

Heart Fields

120. Tbx5 is required in the Second Heart Field for Atrioventricular Septation

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Tbx5 haploinsufficiency causes Holt–Oram syndrome in humans, characterized by atrioventricular septal defects in the heart. We observed atrioventricular septal defects in 40% of late gestation mouse embryos with germline Tbx5 haploinsufficiency. However, no septation defects were observed in embryos with conditional Tbx5 haploinsufficiency in myocardial cells, using Tnt:Cre, or in endocardial cells, using Tie2:Cre, suggesting that the requirement for Tbx5 in cardiac septation may lie outside the heart. Recent work from our laboratory and others has implicated the posterior second heart field in atrial septation. We observed Tbx5 expression in a domain overlapping progenitors for the atrial septum in the posterior second heart field. Furthermore, atrioventricular septal defects were observed in 40% of embryos with conditional Tbx5 haploinsufficiency in the posterior second heart field, using the Hedgehog (Hh) signaling-responsive Gli1:Cre, suggesting that Tbx5 and Hh-signaling are required for atrial septation in the same lineage. We found that Tbx5 and Hh-signaling components genetically interact: Decreasing Hh signaling caused increased penetrance of atrioventricular septal defects in Tbx5 mutant mice. Furthermore, constitutive activation the Hh-signaling rescued atrioventricular septation in Tbx5 mutant mice. Using Genetic Inducible Fate Mapping, we demonstrated that Tbx5 is specifically required for proliferation of Hh-receiving atrial septal progenitors in the posterior second heart field. We conclude that Tbx5 is required in the posterior second heart field, not in the heart, for cardiac septation. We present a molecular pathway including Tbx5 and Hh signaling required for cardiac progenitor cell specification that informs the ontogeny of atrial septal defects.

121. Cooperative roles of *Tbx1* and *Tbx3* during early outflow tract development

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In the early embryo progenitor cells of the second heart field (SHF) contribute to rapid growth of the heart tube. SHF cells in pharyngeal mesoderm are adjacent to pharyngeal ectoderm and endoderm and SHF deployment is concomitant with neural crest cell migration. Tbx1, the major DiGeorge syndrome candidate gene and Tbx3, encoding a transcriptional repressor, are required for outflow tract development. These two genes have complementary expression profiles in the pharyngeal region; Tbx1 is expressed in the lateral pharyngeal endoderm, ectoderm and mesoderm (including the SHF) while Tbx3 is expressed in ventral pharyngeal endoderm and neural crest cells. Tbx3 transcript levels are decreased in neural crest cells in the caudal pharynx of Tbx1 null embryos suggesting that Tbx3 expression may be regulated by Tbx1 dependent signals originating from pharyngeal epithelia or mesoderm. We are investigating potential interaction between these genes by a genetic cross. Pharyngeal arch artery defects in double heterozygous Tbx1 Tbx3 mutant embryos are similar in severity and frequency to those in Tbx1 heterozygous embryos. However, double homozygous Tbx1 Tbx3 mutant embryos have impaired development and display lethality at midgestation. These embryos are characterized by pericardial edema associated with a failure of heart tube elongation and looping. This phenotype is more severe than that of Tbx1 or Tbx3 homozygous mutant embryos suggesting a cooperative role of these two factors during early heart morphogenesis. Altered expression of Tbx2 is also observed in Tbx1 mutant embryos. We are currently analyzing the molecular phenotype of double homozygous Tbx1 Tbx3 embryos.molecular phenotype of double homozygous *Tbx1 Tbx3* embryos.

Neural Crest & Conduction System

Neural Crest

59. Trigenic neural crest-restricted Smad7 over-expression results in congenital craniofacial and cardiovascular defects

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Smad7 is a negative regulator of TGFβ superfamily signaling. Using a three-component triple transgenic system, expression of the inhibitory Smad7 was induced via doxycycline within the NCC lineages at pre- and post-migratory stages. Consistent with its role in negatively regulating both TGFβ and BMP signaling in vitro, induction of Smad7 within the NCC significantly suppressed phosphorylation levels of both Smad1/5/8 and Smad2/3 in vivo, resulting in subsequent loss of NCC-derived craniofacial, pharyngeal and cardiac OFT cushion cells. At the cellular level, increased cell death was observed in pharyngeal arches. However, cell proliferation and NCC-derived smooth muscle differentiation were unaltered. NCC lineage mapping demonstrates that cardiac NCC emigration and initial migration are not affected, but subsequent colonization of the OFT was significantly reduced. Induction of Smad7 in post-migratory NCC results in cardiac OFT anomalies and interventricular septal chamber septation defects, suggesting that TGFβ superfamily signaling is also essential for cardiac NCC at post-migratory stages to govern normal cardiac development. Taken together, the data show that tightly regulated TGFβ superfamily signaling plays an essential role during craniofacial and cardiac NCC colonization and cell survival in vivo.

60. Cardiac Neural Crest Nibble Their Way to the Heart

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Cardiac neural crest ablation causes abnormal development of the secondary heart field with consequent abnormal cardiac looping and malalignment defects of the cardiac arterial pole. Malalignment is caused by excess FGF8 signaling. It is unknown how cardiac crest cells normally decrease FGF8 signaling in the caudal pharynx. We show that cardiac crest cells use a retinoic acid signal to reduce endodermal fgf8 production. In addition, the crest cells use receptor-mediated endocytosis to amass FGF8 protein for degradation. Further, we show that FGF8 is chemokinetic for cardiac crest cells via FGF receptors 1 and 3 and MAPK/ERK intracellular signaling, and that a site of forced overexpression of ectopic FGF8b by the pharyngeal endoderm becomes surrounded by cardiac crest cells. Because of the protective effect of neural crest, ectopic overexpression of FGF8 results in only subtle arterial pole defects, not involving malalignment. Protection of the pharynx from abnormal signaling by endocytosis represents a new role for neural crest cells.

Conduction System

193. Origins of Cardiac Pacemaking Cells in Avians

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Rhythmic heartbeat is controlled by action potentials originating from pacemaking cells of the Sinoatrial Node (SAN). Despite the importance of pacemaker cells for the initiation and rhythmicity of heartbeat, little is known regarding their developmental origins or mechanisms of specification and differentiation. Here we report our direct cell tracing studies, mapping the origins of SAN precursors to a small region in the lateral plate mesoderm posterior to the developing primary and secondary heart fields. These cells take over pacing from primary heart field-derived cells between E2.5-E3 in Chick. Furthermore, they continued to act as the dominant pacing center throughout the dramatic morphogenetic processes of cardiac looping and septation, finally incorporating into the atrium by E12. To address the underlying mechanism of SAN precursor specification and differentiation, the precardiac fields (PCFs) and SAN precursor mesoderm were isolated at HH stage 8 and cultured ex vivo. PCF explants underwent tube formation and looping remarkably similar to hearts in vivo, and displayed spontaneous heartbeat over the first 2 days in culture, however, both beat rate and rhythmicity decreased significantly afterwards. In striking contrast, co-culture of PCF explants with SAN precursor mesoderm resulted in maintenance of rhythmicity through 3 days in culture. Interestingly, SAN precursors when cultured alone displayed increased beat rate and rhythmicity. Collectively, these data suggest that the fate of SAN precursors is specified by HH stage 8 and proper differentiation of these pacemaking cells does not require PCF cells. Supported in part by grants from NIH-NHLBI.

194. The role of Tbx3 and Nkx2-5 in the adult atrioventricular conduction system

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The coordinated contraction of the heart is precisely timed by the cardiac conduction system. The conduction system expresses Tbx3, which is required for the specification of the sinus node and of the atrioventricular (AV) conduction system during development. In human, heterozygous TBX3 mutations cause congenital defects belonging to the ulnar-mammary syndrome. The consequences of heterozygous loss of Tbx3 in the heart, however, are still unknown. Quantitative and 3D morphometrical analysis of AV node and bundle showed a significant smaller AV bundle in adult Tbx3 heterozygous mutants compared to wild-type mice, whereas the size of the AV node was unchanged. Furthermore, functional analysis showed a significant shortening of the AV conduction time in heterozygous Tbx3 mutant mice. Tbx3 has been shown to physically interact with Nkx2-5 to repress target genes in the cardiac conduction system. Similar as in Tbx3 heterozygous mice, the AV bundle was significantly smaller in Tbx3/Nkx2-5 and Nkx2-5 heterozygous mice in comparison to the wild-type situation. In contrast to earlier reports, we found that the size of the AV node was not affected in heterozygous Nkx2-5 mutants. Heterozygous loss of Nkx2-5 or of Tbx3 and Nkx2-5 caused complete AV block in a significant number of mice. Interestingly, also atrial ectopic beats were found in both groups of heterozygous Nkx2-5 mutant mice, suggesting the possibility of expansion of pacemaker cells into the atrium as previously observed in Nkx2-5 hypomorphic embryos. Our data indicate that normal levels of Tbx3 and Nkx2-5 are independently required for AV bundle formation and homeostasis.

195. Delayed atrioventricular conduction in adrenergic-deficient embryonic mouse hearts: Uncovering a role for retinoic acid

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Dopamine-β-hydroxylase (Dbh) is a key enzyme in the adrenergic hormone synthesis pathway and is critical for cardiac development and survival past embryonic day 10.5 (E10.5) in the mouse. We have previously shown that intrinsic cardiac adrenergic cells populate regions of the heart associated with the conduction system. To investigate physiological mechanisms of action for adrenergic hormones in cardiac conduction system development, we evaluated E10.5 adrenergic-deficient (Dbh-/-) and adrenergic-competent (Dbh+/+) mouse hearts cultured on microelectrode arrays (MEAs, 8x8, 200 microns apart). Our results show that although mean heart rates were similar in the two groups, atrioventricular conduction time was significantly slowed in the Dbh-/- hearts when compared to controls (31.4+6.4 vs. 15.4+1.7 ms, respectively; $p < 0.05$). To identify potential molecular pathways mediating this process, we evaluated gene expression profiles of E10.5 Dbh-/- and Dbh+/+ hearts using Affymetrix Mouse Genome 430A 2.0 Arrays. Of the more than 22,000 genes on the array, we found that significant differences of 2-fold or greater magnitude occurred in 22 genes (~0.1% of total). Notably, several of these genes are involved in retinoic acid synthesis and signaling, including: retinol dehydrogenase-12, ubiquinone, beta carotene monooxygenase-1, and fibroblast growth factor-20 (FGF-20). Importantly, retinoic acid signaling has been shown to be critical for heart development during the mid-gestation period, and genetic disruption of retinoic acid receptors has been shown to lead to atrioventricular conduction block. Thus, our results show that adrenergic hormones may influence cardiac conduction system development via regulation of retinoic acid signaling in the embryonic heart.

Cardiomechanics

20. Shear stress modulates transcriptional regulation and cushion endoMT in the developing heart

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Proper cardiovascular development depends on biomechanical stimuli, among which the fluid shear stress exerted by blood flow. Endothelial cells (EC) are particularly sensitive to shear stress and translate this force into functional and phenotypic responses, which include calcium signaling, NO production, and gene expression regulation. The latter is largely coordinated by the transcription factor Krüppel-like factor 2 (Klf2). We described earlier that endocardial Klf2 expression delineates areas of high shear stress in the embryonic heart, and that experimentally altered flow in vivo in the venous clip model leads to changes in expression patterns of Klf2 and downstream targets. Here we describe that the model also presents with a 50% loss of atrioventricular cushion mesenchyme due to a lack of endothelial to mesenchymal transition (endoMT). Since transforming growth factor β (Tgfβ) plays a central role in cushion endoMT we analysed the relation between shear stress, Klf2 expression, and activation of Tgfβ signalling in embryonic endothelial cells. In adult EC shear stress results in downregulation of Tgfβ signalling through Klf2-mediated induction of the inhibitory Smad7. We provide evidence that in embryonic EC Tgfβ signalling through the type I receptor Alk5 is necessary for flow-stimulated induction of Klf2, and leads to a rapid phosphorylation of the receptor Smad2 in vitro and in vivo. The induction of Klf2 could be abrogated with inhibitors for Alk5 and Mek5 kinase activity. Herewith we identify shear stress as an important modulator of cushion endoMT in the developing heart through activation of TGFβ signalling and Klf2.

21. Integrative computational modeling and imaging approach to study the effects of abnormal hemodynamic conditions on cardiac development.

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It is well accepted that hemodynamic forces modulate cardiac growth during embryonic development, with abnormal hemodynamic forces leading to heart defects. However, the mechanisms that regulate cardiac growth in response to hemodynamic stimuli have remained elusive, in part due to the lack of methodologies to accurately assess the forces exerted by blood flow on cardiac tissue. To calculate the forces to which cardiac cells are subjected during the cardiac cycle we are employing a combination of imaging and computational modeling. Our studies use the chick embryonic heart, focusing on the heart outflow tract (OFT) at early stages of development – HH18 to HH24 – when the heart is a looped tube, because at these stages the OFT is very sensitive to hemodynamic forces. We use optical coherence tomography (OCT) to simultaneously acquire structural and Doppler tomographic image sequences of the beating heart in vivo. OCT image sequences are acquired at different locations that together span the OFT. 4D images of the beating heart are then reconstructed and segmented from structural images (using our developed algorithms), and blood flow velocities computed from Doppler OCT images. To determine the influence of wall motion on blood flow dynamics, as well as distributions of shear stresses on the walls of the OFT, we use computational models. We found that curvature, and the distribution of cardiac cushions (protrusions of extra-cellular matrix into the OFT lumen) affect the spatial distribution of blood flow velocities and wall shear stresses, which could lead to differential cardiac growth and remodeling.

22. Knockdown of embryonic myosin heavy chain causes defective electrical activities and calcium signalling within the developing heart

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Our group has previously shown that embryonic myosin heavy chain (eMHC) morpholino knockdown in chicks at HH14 results in aberrant cardiac development including abnormal atrial septation and decreased ventricular wall thickness. Therefore, to provide functional insights the electrical activity and calcium signalling were analysed. Knockdown of eMHC was performed at HH14 (harvested HH19). Electrical activity studies were carried out by intracellular recordings of intact hearts and single cell cultures were incubated with fluorescent dyes to detect intracellular Ca2+ transients. eMHC knockdown hearts exhibited abnormal beating patterns characterized by the absence and/or weak contraction of the ventricle. Although the atria displayed normal contraction, the action potentials (APs) of the cells exhibited decreases in amplitude, maximal rate of rise and increase in duration. In contrast, no AP was recorded in 75-80% of ventricular cells. In the cells that exhibited spontaneous APs, small amplitude, long duration and slow rate of rise was observed when compared with control hearts. Knockdown hearts also showed erratic amplitudes and/or frequencies of [Ca2+]. The atrial cells displayed a large variability in the duration of the Ca2+ transient, including superimposition of a second or third maximal Ca2+ peak, and small or aborted Ca2+ transient peaks. In the ventricular cells, eMHC knockdown induced small changes in Ca2+ transient frequency. This data demonstrates that eMHC plays critical roles during cardiogenesis, specifically with normal ventricular and atrial function. Novel insights into the role that the structural protein eMHC plays in the electrical activity and calcium signalling within the developing heart are provided.

POSTER SESSIONS
THURSDAY 21 MAY- SESSION A

Time: 12.00 - 14.00 hours
Location: Marble Hall
1st floor and basement

Session Topics

Cardiac Signaling	[posters 1 - 19]
Cardiomechanics	[posters 19 - 30]
Epicardium	[posters 31 - 46]
Extra-Cellular Matrix and Morphogenesis	[posters 47 - 58]
Neural Crest	[posters 59 - 68]
Valve Development	[posters 69 - 91]

Session A - Cardiac Signalling

1. Inhibition of Bmp signaling by Smad6 is required for terminal differentiation of ventricular cardiomyocytes.

Emma de Pater (1) and Jeroen Bakkers (1,2)
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For abstract, see in oral presentation section page 48

2. The BMP Pathway Acts to Regulate Tbx20 in the Developing Heart

Erin Kaltenbrun* (1, 2), Elizabeth Mandel (1, 2), Thomas Callis (1, 4), Da-Zhi Wang (1, 4, 5) and Frank Conlon (1, 2, 3)
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For abstract, see in oral presentation section page 49

3. Bmp-signaling regulates myocardial differentiation from cardiac progenitors through a micro RNA-mediated mechanism

Jun Wang* (1) , Margarita Bonilla-Claudio (1), Jue Zhang(1), Yan Bai (1), Zheng Huang (1), Brian L. Black (2), Fen Wang (1), and James F. Martin (1)
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For abstract, see in oral presentation section page 50

4. Cardiac Laterality is Controlled by Multiple Rho Kinase Signaling Pathways in Ciliated Cells

Guangliang Wang, Fiona C. Foley, Duck Soo Jang, Jeffrey D. Amack*
Department of Cell and Developmental Biology, State University of New York Upstate Medical University, Syracuse, NY, USA

For abstract, see in oral presentation section page 51

5. Mitochondrial structure and function during cardiac myocyte differentiation

George A. Porter, Jr.* (1,2,3), Jennifer Hom (1), Rodrigo Quintanilla (1,2), David Hoffman(1), Bentley Karen (4), Shey-Shing Sheu (2)
(1) Department of Pediatrics, (2) Department of Pharmacology and Physiology, (3) Aab Cardiovascular Research Institute, (4) Department of Pathology and Laboratory Medicine, University of Rochester Medical Center, Rochester, NY, USA

For abstract, see in oral presentation section page 52

6. BMP-mediated inhibition of FGF signaling lies at the heart of differentiation

Libbat Tirosh-Finkel (1), Amit Zeisel (2), Miriam Brodt-Ivenshitz (1), Ayelet Shamai (1), Zhong Yao (1), Rony Seger (1), Eytan Domany (2), and Eldad Tzahor* (1)
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The transition from progenitors to differentiated cells is critical for successful organogenesis; alterations in this process can lead to serious developmental disorders. The anterior heart field (AHF) encompasses a niche in which cardiac progenitors maintain their multipotent and undifferentiated nature in response to signals from surrounding tissues. Here we investigate the shift from proliferating cardiac progenitors to differentiating cardiomyocytes in chick embryos. Genomic and systems biology approaches as well as perturbations of signaling molecules, in vitro and in vivo, reveal tight crosstalk between the bone morphogenic protein (BMP) and fibroblast growth factor (FGF) signaling pathways within the AHF: BMP4 promotes myofibrillar gene expression and cardiomyocyte contraction, by blocking FGF signaling. Furthermore, inhibition of the FGF-ERK pathway is both sufficient and necessary for these processes, suggesting that FGF signaling blocks premature differentiation of cardiac progenitors in the AHF. Investigating the molecular mechanisms downstream of BMP signaling revealed that BMP4 induced a set of neural crest-related genes; including MSX1. Overexpression of Msx1 was sufficient to repress FGF gene expression and cell proliferation, thereby promoting cardiomyocyte differentiation. Hence, BMP and FGF signaling pathways act via inter- and intra-regulatory loops in multiple tissues, to coordinate the balance between proliferation and differentiation of cardiac progenitors.

7. Involvement of the MEKK1 Signaling Pathway in the Regulation of Epicardial Cell Behavior by Hyaluronan

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During embryonic development, cells comprising the outermost layer of the heart or epicardium play a critical role in the formation of the coronary vasculature. Thus, uncovering the molecular mechanisms that govern epicardial cell behavior is imperative to better understand the etiology of cardiovascular diseases. In this study, we investigated the function of hyaluronan (HA), a major component of the extracellular matrix, in the modulation of epicardial signaling. We show that stimulation of epicardial cells with high molecular weight HA (HMW-HA) promotes the association of MEKK1 with the HA receptor CD44 and induces MEKK1 phosphorylation. This leads to the activation of two distinct pathways, one ERK-dependent and another NFkB-dependent. Furthermore, HMW-HA stimulates epicardial cells to differentiate and invade, as suggested by increased vimentin expression and enhanced invasion through a collagen matrix. Blockade of CD44, transfection with a kinase inactive MEKK1 construct or the use of ERK1/2 and NFkB inhibitors significantly abrogates the invasive response to HMW-HA. Together, these findings suggest an important role for HA in the regulation of epicardial cell fate via activation of MEKK1 signaling cascades.

8. Wnt/ß-catenin signaling and miRNAs regulate Notch signaling in heart morphogenesis

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In this study, we ablated the Wnt effector ß-catenin, the Notch mediator RBPJ and the miRNA processor Dicer in the developing heart of mice using MesP1-cre, which acts in mesoderm progenitors that contribute to first and second heart field (FHF, SHF). Conditional ablation of ß-catenin by MesP1-cre affected cardiac looping and right ventricle formation due to defective expansion of Isl1-expressing SHF progenitor cells. Moreover, loss of ß-catenin promotes the expression of Notch target genes, indicating that ß-catenin controls the level of Notch signaling in the developing heart. In MesP1-cre; RBPJ lox/lox mutants, the formation of the right ventricle as well as the expression of the right ventricular cardiomyocyte marker dHand were affected. Remarkably, the expression of Gata4 and Mef2c in SHF derivatives was abolished and we detected increased cellular death. Our data indicate that Notch signaling promote the differentiation and survival of SHF cells required for SHF derivative formation. Although Notch signaling has previously been shown to be essential for heart development, only little is known about the spatiotemporal regulation of Notch signaling in SHF cells. Emerging evidence suggest that miRNAs can function as regulators of Notch signaling in various tissues. Interestingly, in MesP1-cre; Dicer lox/lox mutants the expression of Notch ligand and target genes, like Jag1, Hrt2, Mef2c, dHand is elevated in SHF cells. Together, these results suggest a Wnt/ß-catenin-Notch crossregulation in heart formation possibly involving the activation of specific miRNAs or Notch inhibitors.

9. Identification of Thymosin Beta 4 Protein Interactors Using a Yeast Two-Hybrid Screen

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Thymosin beta 4 (Tβ4), a 43 amino acid peptide, plays an important role in cardiac, neural and gastrointestinal tract development. In the embryo, Tβ4 knockdown studies have demonstrated an essential requirement for Tβ4 in coronary vasculogenesis and in systemic vasculature development. In addition, Tβ4 has numerous roles in biological processes including, but not limited to, inflammation, wound healing, cardioprotection, regeneration, angiogenesis, metastasis, and hair follicle growth. The developmental role of Tβ4 is recapitulated in the adult, inducing neovascularization post myocardial infarction. Thus, Tβ4 is of considerable interest due to its potential in cardiac regenerative therapy. As well as its prominent role in actin polymerization, Tβ4 has been postulated to regulate proteases, cytokines, chemokines, matrix molecules, survival kinases and inflammatory molecules. Yet, the precise molecular mechanisms through which Tβ4 acts in signal transduction remain equivocal. An intracellular actin-binding role for Tβ4 is well established. Both in vitro and in vivo studies show strong evidence that Tβ4 also acts extracellulary through paracrine signalling. Moreover there is evidence that Tβ4 is internalized. To help understand the precise mechanisms through which Tβ4 functions we are performing a yeast two hybrid screen using full length Tβ4 cDNA as a bait to identify potential Tβ4 regulatory proteins from an embryonic day 11 library. Deciphering Tβ4 signal transduction pathways will advance our understanding of its role in normal human development and help unlock its full therapeutic potential.

10. Loss of Sphingosine-1-Phosphate Receptor S1P1 Disrupts Cardiac Development.

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Sphingosine-1-phosphate (S1P) is a biologically active sphingolipid metabolite that influences numerous cellular events including differentiation, proliferation and migration. S1P acts through cell surface receptors S1P1-5, with S1P1 having the highest expression level in the developing heart. S1P1 signaling is critical for vascular maturation, however its role during cardiac development is not well known. To determine the role of S1P signaling during heart development, we performed morphological analysis on S1P1 and S1P3 knockout mice. Examination of E10.5 S1P1-/- embryos revealed that although the embryos were not smaller than S1P1+/+ and S1P1+/- littermates, the hearts were smaller and malformed. Hearts of S1P1-/- embryos have shortened AV canal cushions and irregular trabeculae. However, S1P3-/- embryos examined at E10.5 showed no differences in the overall size of the embryos or in heart morphology among the different S1P3 genotypes. At E12.5, although no difference in embryo size was observed between the different genotypes, S1P1-/- hearts were smaller with the apex of the heart rounded compared to a normal V-shape for S1P1+/+ hearts. The average length of the S1P1-/- hearts was 22.7% less than the S1P1+/+ hearts when measured from the AV canal to the apex of the heart. In addition, the S1P1-/- hearts had 21% less ventricular myocardial tissue than S1P1+/+ hearts. Immunostaining with the cardiac marker alpha-sarcomeric actin revealed disrupted morphology of the heart wall and trabeculae, with a thickening of the outer compact layer. This report indicates that S1P signaling through the S1P1 receptor is critical for cardiac development.

11. Microcirculation alteration in aged-hearts

Tania Zaglia*(1), Valentina Mazzariol (2), Silvia Mainente (2), Mayan Seri (2), Tullio Pozzan (1,2), Stefano Schiaffino (1,2) and Marco Mongillo (1)
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Introduction: The cardiac Sympathetic Nervous System (SNS) modulates coronary vasodilatation, heart frequency and contractility. A functional impairment of SNS occurs with aging and contributes to the pathophysiology of several cardiovascular diseases. However, it is not yet clear whether the interactions between sympathetic terminals (STs) and target cells change with aging and whether these alterations affect myocardial morphology and functionality. Aims of the study: We aimed to: i) provide a morphological description of the interaction between STs, vasculature and cardiomyocytes; ii) investigate how this relationship changes with aging and the functional consequences of such alteration. Results: Using immunofluorescence analysis we demonstrated that in normal adult mouse hearts STs are distributed at each anatomical level with a greater density in the atria and the right ventricle. STs were found in proximity to coronary vessels, cardiomyocytes and to capillaries. Similar analysis on hearts from aged mice demonstrated a significant reduction of sympathetic myocardial innervation with morphological alterations of STs. STs were shorter and fragmented in aged hearts. In addition, hearts from aged mice showed cardiomyocyte atrophy, increased capillary density and reduced capillary size. Sympathectomized adult hearts reproduced the same phenotype observed in aged hearts, with about a 20% decrease in cardiomyocyte size, as compared to controls, and altered capillary organization. Conclusions: The structural and functional integrity of SNS is crucial in the maintenance of cardiomyocte trophism and capillary organization in the postnatal mouse heart. Future development of these studies will aim to determine the molecular pathways involved in the aging-related SNS impairment.

12. Gene Profiling In Hgf-Stimulated Early Postnatal Heart.

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We investigated the role of HGF in cardiomyocytes in vivo, by using animals with cardiac-specific HGF overexpression and full genome DNA microarray analysis. We performed microarray analysis of mouse heart at two distinct developmental ages (postnatal days P2 and P7), which correspond to the transition between the plastic and post-plastic phase. When comparing HGF-expressing hearts versus wild-type hearts at P7 we found ~200 genes significantly upregulated more than 1.7-fold. Several of these genes play a role in cell cycle regulation, translation, signal transduction, transcriptional regulation, cytoskeleton/extracellular matrix and membrane vescicle traffic. The majority of these genes were downregulated from day 2 to day 7 in the wild-type heart. Thus, gene upregulation induced by HGF opposed at physiological downregulation. We confirmed that in vivo treatment with HGF increased Ki67 expression by 3-fold during day 7, indicating that HGF prolongs the plastic phase in neonatal mouse heart. As adults, the mice with cardiac-specific HGF overexpression develop a systolic contractile defect, even when HGF expression is suppressed after birth. Conclusions: The analysis of global gene expression in the whole heart may be useful in understanding the orchestrated process of postnatal development or terminal differentiation in the cardiac environment. These data are likely to be helpful in studying the Developmental Origins of Cardiac Disease.

13. Pro-hypertrophic signaling mechanisms downstream of mutant Shp2 associated with Noonan / LEOPARD Syndrome

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The protein tyrosine phosphatase Shp2 is essential for normal prenatal development. In mice, complete deletion of Shp2 protein is embryonic lethal. In humans, loss-of-function (LOF) mutations in Shp2 are primarily found in Noonan or LEOPARD Syndrome families. These overlapping syndromes are characterized by congenital heart defects such as pulmonary stenosis and hypertrophic cardiomyopathy (HCM). Unfortunately, the underlying disease mechanisms in particular with regard to HCM are not clearly understood. In normal cardiomyocytes, Shp2 dephosphorylates focal adhesion kinase (FAK) resulting in reduced signaling through mammalian target of rapamycin (mTOR), thus limiting cell size. We hypothesize that mutant Shp2 fails to down-regulate FAK-related signaling and thereby stimulates cardiomyocyte growth. Our studies focused on the LOF mutation Q510E-Shp2, which causes a particularly aggressive form of HCM in humans. We examined the influence of Q510E-Shp2 on cell size in cultured neonatal cardiomyocytes. Adenoviral expression of Q510E-Shp2 resulted in a 40% increase in average cell area. The FAK / src inhibitor PP2 blocked Q510E-Shp2's effect. Similarly, co-expression of the endogenous FAK inhibitor FRNK abolished the pro-hypertrophic effect of Q510E-Shp2. Rapamycin, but not the PI3kinase inhibitor LY294002, also prevented the Q510E-Shp2-induced cell area increase, showing that mTOR is a specific mediator downstream of FAK. Interestingly, the MEK1 inhibitor U0126 also attenuated the cell size increase due to Q510E-Shp2 expression. This indicates at least a partial role for MEK1/ERK signaling either parallel to or downstream of FAK. In summary, Q510E-Shp2 acts as a potent stimulator of cardiomyocyte growth mediated by signaling through FAK / mTOR and MEK1/ERK.

14. Notch Signalling in Cardiogenesis: 3D Image Analysis

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Notch is a local cell signalling system that regulates cell fate specification and tissue patterning during development and adult tissue homeostasis. We are carrying out a 3D image analysis of Notch expression in the developing heart, to get an insight into the mechanism of Notch signalling activity in this organ. We are also trying to determine which elements are crucial for signaling during early heart development, focusing in the processes of trabeculation and valvulogenesis. Our hypothesis, attending to our initial results, is that the activation pattern of the Notch1 receptor is not only due to the action of the Delta4 ligand as during trabeculae formation, other Notch ligands or modulators may restrict Notch1 activation. In addition, expression of the Jag1 ligand in the myocardium and the absence of any Notch receptor in this tissue, suggests a possible intercommunication between endocardium and myocardium that may pattern Notch activity in the developing chamber. Functional analysis will determine which ligands or modulators are important for the Notch signalling during the early stages of heart development.

15. Platelet-Derived Growth Factor Receptor β is Critical for Zebrafish Intersegmental Vessel Formation

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Coronary heart disease is among the leading causes of disability and mortality in the United States and worldwide. In contrast to humans, zebrafish hearts fully regenerate after amputation of up to 20% of the ventricle. During this regeneration process, new coronary blood vessels form to revascularize the is a)regenerating tissue. Platelet-derived growth factor receptor β (PDGFRβ tyrosine kinase receptor known to affect vascular development. The zebrafish is an excellent model to study specific regulators of vascular development and regeneration, yet the role of PDGF signaling has not been determined in zebrafish embryos or during adult zebrafish heart regeneration. Furthermore, vascular mural cells, in which PDGFRβ functions cell autonomously in other systems, have not been identified in zebrafish embryos younger than 72 hours post fertilization. To investigate the role of PDGFRβ in zebrafish vascular development, we cloned the highly conserved zebrafish homolog of PDGFRβ. We found that pdgfrβ is expressed in the dorsal aorta, posterior cardinal vein, and intersegmental vessels of the developing zebrafish vasculature. A similar expression pattern was observed for pdgf-b, the predominant ligand for PDGFRβ. Using a PDGFR tyrosine kinase inhibitor and a morpholino oligonucleotide specific to PDGFRβ, we found that PDGFRβ is necessary for angiogenesis of intersegmental vessels while having no role on formation of the dorsal aorta or posterior cardinal vein. Our data provide the first evidence that PDGFRβ signaling is required for zebrafish developmental angiogenesis. We propose a novel mechanism for zebrafish PDGFRβ signaling that regulates vascular angiogenesis in the absence of mural cells.

16. Role of Hedgehog signaling on the developing avian vascular system

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The first blood vessels in the embryo are formed through the process called vasculogenesis. This process involves assembly of angioblasts (vascular endothelial precursor cells) in to a loose cord, followed by establishment of close cell contacts and formation of a vascular lumen. Within the developing avian embryo, the dorsal aorta is the first major blood vessel to form. Lumenization of the dorsal aorta occurs at approximately the 7-9 somite stage (Hamilton and Hamburger (HH) stage 9) of development. A rostral-caudal progression is observed during this process, where the anterior part of the dorsal aorta shows a tube morphology while the posterior portion is a cellular cord. Previous work in our lab has shown a role of the growth factor sonic hedgehog (Shh) during blood vessel formation. Own initial conclusions are that Hedgehog (Hh) is required for vascular remodeling (angiogenesis) but not vascular tubulogenesis. Inhibition of Hedgehog pathway by a hedgehog inhibitor, cyclopamine, caused irregular dorsal aortae and a discontinuous vascular plexus without alter the endothelial cell proliferation in vivo. In addition Hedgehog pathway is necessary to form the intersomitic vessels. Consistent with this result, use of Smoothened Agonist (SAG) shows that activation of Hedgehog is able to induce vascular growth and amount of fenestration. These effects are independent of Notch and Bone Morphogenetic Proteins 4 (BMP4) signaling. However Hedgehog signaling is able to regulate VEGF expression. These studies contribute to our understanding of Hedgehog's role in vascular development.

17. PDK1 regulates cardiomyocyte proliferation, epithelial-mesenchymal transition and chamber formation in heart development

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One essential downstream signaling pathway of receptor tyrosine kinases such as VEGFR and Tie2 receptor, is the PI3K-PDK1-Akt/PKB cascade that plays a critical role in development and tumorigenesis. However, the role of PDK1 in cardiovascular development remains unknown. Here, we deleted PDK1 specifically in cardiac progenitors, endothelial cells and the second heart field, respectively in mice. These mice displayed cardiac hypoplasia, hemorrhage and hydropericardium, and defective chamber formation, and died from E9.5 to E14.5. Histological analysis revealed reduced cardiomyocyte proliferation, defective vascular remodeling and development, and disrupted integrity between endothelium and trabeculae/myocardium in the heart. The atrioventricular canal (AVC) cushion and valves failed to form indicating a defect in endothelial-mesenchymal transition (EMT). Consistently, ex vivo AVC explant culture showed impeded mesenchymal outgrowth. Increased apoptosis was found in endothelial cells. Snail protein was reduced and was absent from nucleus in AVC cells. Furthermore, adenoviral Akt delivery and dominant-negative Snail rescued EMT defects in AVC explant culture. Deletion of Pten rendered normal development of AVC cushion in PDK1 deficient heart by E11.5 and survival of the mice. Taken together, these results have revealed an essential role of PDK1 in cardiovascular development through activation of Akt.

18. Conditional ablation of Hif1α in the mouse reveals new insight into coronary vascular development

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HIF-1α is a basic helix-loop-helix (bHLH)-PAS family transcription factor that is rapidly degraded under normoxic conditions. In hypoxia, degradation of HIF-1α is inhibited, allowing accumulation in the cytosol and translocation to the nucleus where along with ARNT (Hif1β) transcriptionally regulates genes mediating processes including blood vessel maintenance and growth. Hif1α is known to play a role in cardiac and blood vessel development during embryogenesis. Homozygous null mutants for Hif-1α die by embryonic day 10.5 (E10.5) with severe cardiovascular defects. However, this lethal phase has precluded investigation into Hif1α's functions during later heart development. Here we tested the hypothesis that Hif-1α plays critical roles in development of the coronary circulation, which occurs after E10.5. To carry out this study we used cell specific expression of Cre to ablate an allele of Hif-1α containing loxP sites flanking exon 2, which contains the bHLH domain. We used Wt1-Cre to induce recombination in epicardium and Myh6-CreESR to induce recombination in myocardium. To avoid the early requirements of Hif1α in myocardium, we activated Cre after E9.5 with tamoxifen. When Hif1α was ablated in myocardial cells we observed alterations to the formation of blood islands on the surface of the heart at E13.5. Here mutants had larger, flattened blood filled lumens, compared to Cre- littermates, which had spherical, protruding blood islands. When Hif1α was ablated in epicardial cells we observed alterations to vessel wall thickness in weaned juvenile mice, compared to littermates. Together, these findings indicate that Hif1α performs distinct functions in myocardium and epicardium to regulate different phases of coronary vessel development.

19. CHAP, a calcineurin regulated protein essential for striated muscle function

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In recent years, the perception of Z-disc function has changed from a passive anchor for myofilaments that allows transmission of force, to a dynamic multicomplex structure, capable of sensing and transducing extracellular signals. Since heart failure is the number one cause of mortality and morbidity worldwide, the identification of new components, the precise molecular mechanisms of the Z-disc and its role in signalling has become critical for understanding the regulation of muscle function and disease. We described a new Z-disc protein, which we named CHAP (cytoskeletal ¬heart-enriched ¬¬actin-associated protein), expressed in differentiating heart- and skeletal muscle. Interestingly, in addition to its sarcomeric localisation, CHAP was also able to translocate to the nucleus. Knockdown of CHAP in zebrafish resulted in aberrant cardiac and skeletal muscle development and function. These findings suggest that CHAP is a critical component of the sarcomere with an important role in muscle development. Since Z-disc proteins are often associated with heart disease, we investigated whether CHAP is regulated in cardiac hypertrophy. Importantly, activation of calcineurin, a central modulator of cardiac hypertrophy and disease, led to translocation of CHAP from the Z-disc to the nucleus in cultured embryonic mouse cardiomyocytes. CHAP expression was dramatically

upregulated in transgenic mice expressing constitutively active calcineurin in the heart. Furthermore, CHAP nuclear localization was associated with the transcription factor NFATc2, a downstream mediator of calcineurin signalling. Our data suggest that CHAP may be involved in transcriptional regulation of striated muscle development and disease and represents a candidate gene for genetic (cardio-) myopathies.

Session A - Cardiomechanics

20. Shear stress modulates transcriptional regulation and cushion endoMT in the developing heart

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21. Integrative computational modeling and imaging approach to study the effects of abnormal hemodynamic conditions on cardiac development.

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22. Knockdown of embryonic myosin heavy chain causes defective electrical activities and calcium signalling within the developing heart

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23. Actinin-Associated Protein Palladin contributes to the Development of Normal Embryonic Ventricular Chambers

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a-Actinin-associated cytoskeletal proteins are involved in the developmental pathway for ventricular cardiomyopathy. Palladin is an a-actinin-associated cytoskeletal protein localized at stress fibers, focal adhesions, and other actin-based structures. It plays important roles in actin stress fiber formation, cell adhesion and migration. It is also expressed in the embryonic heart and downregulated in the adult heart, however, its role in the myocardium is unknown. Here we compared the development of embryonic hearts from wide-type (Palld+/+) and palladin-knockout (Palld-/-) mice as well as analyzed the characteristics of embryonic cardiomyocytes (ECMs) from these two type hearts. We found that Palld-/- mice died around E12.5-15.5 with ventricular chamber dilation and wall thinning. These alterations were associated with impaired cell contraction. We then analyzed potential mechanisms underlying the altered contractility. The data reveal a novel role for the cytoskeletal protein palladin in the organization of myofilaments with consequences on the development of ventricular chambers and contractile function (Grants: the Major State Basic Research Development Program of China (2007CB512100, 2006CB0F0900), and the National Natural Science Foundation of China (30871420).

24. Optical Coherence Tomography of the Looping Embryonic Heart: Mechanosensing Mechanisms

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Rationale: Interrelationships between the endocardium, myocardium and cardiac jelly compartments during heart looping have been difficult to resolve due to limitations of technologies to visualize the beating embryonic heart. These limitations have been overcome by 4D- optical coherence tomography (OCT). Objective: In this study using proteomics to identify cell proteins in avian looping hearts and 4D-OCTcombined with other imaging modalities, our study addressed mechanotransducing mechanisms in the developing heart. Methods and Results: Using 3-D image reconstructions, the localization of fibronectin (FN), tenascin-C, alpha-tubulin, and nonmuscle myosin-II (NMHC-II) deposition were correlated with structures detected by dynamic OCT data. Another new imaging modality, optical coherence microscopy (OCM), was used to visualize detailed cardiac architecture of the mouse heart. Our results show: cell processes/fibrils are radially oriented within the cardiac jelly (CJ) connecting the endocardium with myocardium. Cyclical deformations of the endocardium are likely to stretch and create stress on these cell processes/ fibers during the cardiac cycle. The cells/fibers are positioned to transduce strains into biochemical changes. A CJ cell network forms at sites defining ventricular trabeculation post-looping. Lastly, the myocardium during looping slides along the enveloping splanchopleural membrane and the associated cardiomyocyte microcilia are positioned to transduce the external forces into myocyte responses. Conclusions: On the external surface of the myocardium of the beating embryonic heart and on the surface facing the CJ, cellular structures and networks exist whose deformations during the cardiac cycle are likely to be involved in translating biophysical forces into signals for cardiomyocyte differentiation and organ morphogenesis.

25. Type III TGFβ Receptor Heterozygous Null Mice have Abnormal Cardiac Function

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The Type III TGFβ receptor (TGFβR3) binds TGFβ and several Bone Morphogenic Protein (BMP) ligands. Several lines of evidence suggest TGFβR3 plays a unique and nonredundant role in signaling. Tgfbr3-/- mice have double outlet right ventricle, ventricular septal defects, and a thinned myocardium. Embryos die at E14.5 due to failed coronary vessel development. Tgfbr3+/- mice display no overt phenotype. Given the pervasive role of TGFβ and BMP in the regulation of development and tissue homeostasis, we asked whether Tgfbr3+/- mice have a cardiac phenotype. We performed unsedated echocardiograms on 3 month old Tgfbr3+/+ and Tgfbr3+/- littermates; the Tgfbr3+/- mice had significantly decreased fractional shortening (FS) 43.1% ± 0.8 and ejection fraction (EF) 80.7% ± 0.7 (n=8) compared to their Tgfbr3+/+ littermates 48.8% ± 1.0 and 85.7% ± 0.8 (n=8) ($p=0.001$ for FS, $p<0.001$ for EF; normal FS>45% for unsedated mice in our cardiovascular core). We next demonstrated that this phenotype existed in mice as early as 6 weeks of age, with FS 40% ± 0.4 and EF 77% ± 0.5 (n=6) in Tgfbr3+/- mice versus FS 47.3% ± 1.3 and EF 84.5% ± 1.1 (n=5) in Tgfbr3+/+ littermates ($p<0.001$ for FS and EF). The Tgfbr3+/- mice did not have significant chamber dilation or display significant differences in wall thickness, heart rate, or blood pressure when compared with Tgfbr3+/+ littermates. Our data demonstrate that Tgfbr3+/- mice have altered cardiac function, as measured by decreased fractional shortening and ejection fraction, that is not associated with hypertrophy or chamber dilation.

26. Myocardin-related transcription factor B regulates mechanical stress-induced cardiac hypertrophy.

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Myocardin-related transcription factors (MRTFs) are cofactors of serum responsive factor (SRF) which is a crucial transcription factor of cardiac and skeletal muscle differentiation and development. MRTFs respond to extracellular stimuli through RhoA / Rho kinase pathway, and translocate to the nucleus to activate SRF-dependent transcription. It is known that MRTF-B plays roles during heart and outflow tract development, however, its function in adult heart is still unknown. Increased mechanical stress or pressure overload induces cardiac hypertrophy and reactivation of the so–called fetal gene program, which is very similar between fetal and failing hearts. To examine if MRTF-B responds to mechanical stress in adult failing heart, we performed Transverse aortic constriction (TAC) surgery in wild type and MRTF-B mutant mice. As results, we identified MRTF-B mutant mice had a greater cardiac hypertrophic response to TAC than wild type mice, which was accompanied with cardiac fibrosis. Hypertrophic marker genes, such as ANF, BNP, were increased. Furthermore, we observed that MRTF-B translocated to the nucleus by TAC within an hour, and it lasted four weeks. The colocalization ratio of MRTF-B with nucleus in left ventricular cardiomyocyte rose about twice after TAC. These results indicate that MRTF-B responds to mechanical stress and translocates to the nucleus in adult heart, which regulates gene expression and would be important for maintaining heart against pressure overload.

27. The sarcomeroskeleton: a basic framework for myofibril assembly?

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Actin and myosin filaments are arranged to the paracrystalline sarcomeres in striated muscle. Two prominent transverse structures can be distinguished in the sarcomere, the Z-disc at its borders and the M-band in the middle. We have suggested previously that a basic cytoskeletal framework is required for the precise assembly, which consists of sarcomeric alpha-actinin at the Z-disc, myomesin at the M-band and titin filaments spanning in-between and have termed this framework the "sarcomeroskeleton". Using RNAi to knock down alpha-actinin and myomesin in long-term cultures of neonatal rat cardiomyocytes (NRC) we can discern two phenotypes: Alpha-actinin knockdown has little effect on the Z-discs also in long-term cultures and only seems to affect the premyofibril regions, this is partly due to upregulation of non muscle alpha-actinin isoforms. Knockdown of myomesin expression has dramatic consequences for myofibril integrity. Our experiments show that this effect depends on the composition of the M-band as well as on beating activity. Taken together our results demonstrate that the M-band of the myofibril is the major focal point of the sarcomere that has to deal with the management of forces that arise from active contraction, while the Z-disc probably plays a more passive, structural role.

28. Qualitative and quantitative analyses of the morphological-dynamics of early cardiac pump action using video densidometry and optical coherence tomography (OCT)

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During the initial phase of its pump action, vertebrate embryonic hearts are seen as valveless tubular pumps. It was traditionally thought that these tubular hearts generate unidirectional blood flow via peristalsis. Recently, however, the pumping mechanism of early embryonic hearts has become a matter of dispute. Uncovering of the pumping mechanism of tubular embryonic hearts requires detailed information about the hemodynamics as well as morphological dynamics of their pump action. We have therefore analyzed the morphological dynamics of cardiac pump action in chick embryos (HH-stages 16-18) in shell-less culture using densidometric analyses of high-speed video recordings and by M-mode optical coherence tomography (OCT). The high-speed video camera and the OCT probe were integrated into an environmental chamber, which provided stable physiological conditions during examination of the embryos. We have recorded striking differences in the contraction behavior (e.g. contraction speed, duration of systolic occlusion of heart lumen) of the embryonic heart segments (common atrium, AV-canal, embryonic ventricles, outflow tract). Moreover, we show, for the first time, the pump action of tubular embryonic chick hearts in frontal views, which provide a much better understanding of early cardiac pump action than the traditional right or left lateral views.

nce tomography (OCT)

29. Changes of hemodynamics in early quail embryos after acute hypoxic exposure

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During vertebrate embryonic development, regions of the embryo are hypoxic, which contributes to the normal development of the cardiovascular system. Perturbation of the oxygen concentration, therefore, can cause abnormal development of the cardiovascular system. Previous efforts were mostly focused on its long-term effect on the structure of the heart, the patterning of the vessels, and the expression changes of genes related to cardiovascular development. However, it is recently demonstrated that mechanical forces exerted by blood flow plays important roles in early cardiovascular development. We hypothesize that short-term exposure to hypoxia could affect these mechanical forces, and in turn affect the cardiovascular system. Using optical coherent tomography (OCT), we obtained M-mode Doppler images from the vitelline arteries of quail embryos at stage 20 (72 hours after incubation). Three different hypoxia levels (5%, 10%, and 15% O2) were applied during ex ovo culture for 1 hour, and a significant increase of pulsatility index (PI) was observed after only 5-10 minutes of exposure. The increase of PI is the result of the increase of duration and extent of retrograde blood flow in the vitelline arteries. With B-mode Doppler OCT, we also observed similar increase of PI in the outflow tract of the heart. Prolonged exposure (30 minutes) to moderate or severe hypoxia also causes arrhythmia (bradycardia and skipping beats), which follows a different time-course suggesting that it is independent from the increase of pulsatility index. These findings demonstrated that hypoxia could change the hemodynamics in the short term, and potentially affect heart development in the long term.

30. Cardiac Myosin is Required for Proper Sarcomere Assembly

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Though dynamic by nature, actin filament lengths are highly regulated within the context of the cardiac sarcomere. What is the basis for such uniformity? Several mechanisms have been proposed to regulate sarcomeric actin dynamics, all of which involve interactions with other proteins to regulate thin filament length. Studies to rigorously test these models have mostly relied on cell culture. Animal models such as mouse knock outs do exist for certain sarcomeric proteins; however, many of these studies are hindered either by multiple isoforms for a particular protein or early embryonic lethality. In the context of myosin heavy chain, the Xenopus system bypasses both these limitations. Xenopus embryos are able to survive for days in the absence of a functional heartbeat, and only one myosin heavy chain isoform (Myh6) is expressed in the developing heart. We are using standard morpholino methods to block translation of Myh6 and thus knock out myosin expression. Xenopus embryos without Myh6 fail to develop a heartbeat. Embryos are still able to swim normally, indicating that skeletal muscle is unaffected. Most importantly, embryos lacking cardiac myosin heavy chain have disorganized z-lines and disregulation of thin filament length. Our studies argue that myosin thick filament assembly is required both for proper z-line assembly and for regulation of actin dynamics. We will confirm our results in a true genetic background using muzak frogs, which possess a nonsense mutation in the Myh6 gene.

Session A - Epicardium

31. Epicardial fate and Notch signaling

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32 Loss of the Type III Transforming Growth Factor-β Receptor decreases Hyaluronic Acid responsiveness in Epicardial Cells

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33. NFATc1 in epicardium-derived cells is required for fibrous skeleton and intramyocardial coronary vessel development

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34. PDGF Signaling is Required for Epicardial Functions and New Blood Vessel Formation in Regenerating Zebrafish Hearts

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35. PDGFR signaling in the epicardium is required for epicardial EMT and coronary VSMC and cardiac fibroblast development

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36. VEGF-C regulates nuclear localization of Prox-1 in epicardial cells

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We previously demonstrated that adult rat and embryonic mouse epicardial cell lines and primary cultures of avian epicardial cells express the lymphatic markers Prox-1, LYVE-1, and VEGFR-3 in a subset of cells (Karunamuni et al., 2009). We also observed that in WT1-cre/ROSA mice, epicardial cells and their descendants express lymphatic markers in situ. In this study, we explored the mechanisms that may push these pluripotent epicardial cells into the lymphatic phenotype. Adult rat epicardial cells (ARECs) treated with the lymphangiogenic growth factor VEGF-C, underwent a dramatic increase in the level of nuclear-localized Prox-1 and phosphorylated ERK. The accumulation of Prox-1 in the nuclei occurred within minutes and appeared to be factor-specific because it was not observed after FGF or VEGF-A treatment. The MEK inhibitor UO126 that inhibits the phosphorylation/activation of ERK induced a reduction of Prox-1 in the cell nuclei in both untreated and VEGF-C treated cells, suggesting that phosphorylated ERK may regulate Prox-1 nuclear translocation. VEGF-C application in embryonic heart explant cultures induced Prox-1 nuclear expression within 30 minutes in a subset of epicardial cells at the atrioventricular sulcus. Genes potentially downstream of Prox-1 in a VEGF-C activated epicardial cell line were identified by ChIP-Seq, in which DNA fragments containing binding sites for Prox-1 were immunoprecipitated and sequenced. We propose that the lymphangiogenic growth factor VEGF-C binds to VEGFR-3 and acts via the ERK pathway to stimulate the translocation of Prox-1, considered to be a “master lymphangiogenic gene”, into the nuclei of epicardial cells.

37. Retinoic Acid Signaling Regulates Ventricular Compact Zone Formation through Induction of Hepatic Erythropoietin

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Epicardial retinoic acid (RA) signaling has been implicated in the formation -/- αof the compact zone myocardium. In this study we used Raldh2-/- and RXR embryos to clarify the role of RA signaling in this developmental process. We mutants areαshow that the morphological heart phenotypes of the Raldh2 and RXR very similar, characterized by a prominent defect in compact zone growth. Examination of the RARE-lacZ RA-reporter transgene revealed that active RA signaling in cardiac tissues is severely diminished in Raldh2 mutants but not mutant embryos. We concluded that RA signaling inαsignificantly altered in RXR the epicardium is dispensable for compact zone formation and that RA-mediated target gene transcription in non-cardiac tissues is required for this process. RT-PCR and ChIP analysis showed that erythropoietin (EPO) is a valid RA target gene in hepatic tissues. It is known that genetic loss of EPO function impairs compact zone formation although the glycoprotein hormone is not expressed in the heart. We are currently investigating downstream targets of epicardial EPO signaling. Taken together we present a new mechanism for compact zone formation. Paracrine RA signaling in hepatic tissues induces EPO secretion which then binds to its cognate receptor in the epicardium. In turn EPO signaling activates epicardial mitogen production and cardiomyocyte proliferation.

38. Epicardial fate and Notch signaling

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Lineage-tracing studies in chick demonstrated that epicardial cells give rise to endothelial and support cells of the coronary vasculature, and cardiac fibroblasts. Genetic fate mappings in the mouse have recently suggested that a substantial fraction of ventricular cardiomyocytes may also be of epicardial origin. This finding is relevant in the light of experiments that showed that the epicardium represents a cellular source for revascularization during cardiac regeneration in the zebrafish. It is therefore an exciting challenge to search for signaling pathways that mediate epicardial differentiation and to exploit their potential in the wounded adult mammalian heart. Given the controversy on epicardial cell fates in mammals, we reevaluated this problem by using a genetic lineage tracing system in the mouse. The Tbx18 gene is expressed in the left ventricle and ventricular septum, and the epicardium. Thus, a Cre Knock-in into the Tbx18 locus allows to trace the fate of epicardial cells overlying the right ventricle in mice double heterozygous for Tbx18Cre and a suitable reporter gene. Double immunofluorescence analysis of differentiation markers and a sensitive GFP reporter did not detect any right ventricular cardiomyocytes to be of epicardial origin. We will describe the cell types to which epicardial cells were found to contribute using this lineage system. Since Notch signaling has been implicated in vascular development, we wanted to explore the role of this pathway in epicardial differentiation and coronary vessel formation. We used both conditional Tbx18Cre mediated loss-of-function and gain-of function strategies. We will report on our findings that Notch signaling is required and sufficient for differentiation of certain cell lineages of the embryonic epicardium in the mouse.

39. Differential contribution of epicardial derived cells during vertebrate coronary vessel formation

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Coronary vessels arise during cardiac development to meet the increasing metabolic demands of the developing myocardium. As in any other vascular bed, the coronary vasculature is composed by two different kinds of vessels, namely arteries and veins. Different studies have proposed the epicardium or the sinus venosus endocardium as the source of the coronary vasculature progenitors. In this study we combine a variety of cell tracing techniques, in vitro cultures and protein and mRNA expression methods. We suggest that epicardial derived cells give rise preferentially to coronary arteries through a vasculogenic process, while the outline of the venous component of the coronary vasculature is formed by angiogenesis from the sinus venosus endocardium. Evidences are provided on the specific, autonomous gene regulation of coronary arterial versus venous differentiation by ephrinB2 and COUP-TFII.

40. Epicardium-derived progenitor cells: tools towards cardiovascular drug discovery?

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The transplantation of bone marrow stem cells in clinical trials on patients following myocardial infarction (MI) has, thus far, resulted in only modest improvement in cardiac function. Hence, there continues to be an urgent need to identify the most promising cardiovascular stem cells for achieving therapeutic regeneration. A major hurdle in implementing a cell-based regenerative therapy is the inefficient differentiation of cardiomyocytes or coronaries from either endogenous or exogenous stem cell sources. A novel approach to increase cardiovascular cell yields is the use of small molecules to stimulate proliferation, migration, differentiation and maturation, either in cultures prior to cell transplantation or acting directly on resident cardiac progenitors. Here we report the preliminary findings arising from an unbiased chemical genetics screen on both embryonic and adult epicardium-derived progenitor cells (EPDCs). We identify two phenotypic hits in lineage-traced EGFP+ EPDCs induced to form FAP1/alphaSMA+ myofibroblasts (embryo) and a PECAM+ vascular plexus (adult) by classes of compounds previously reported to be cardiogenic(1). Myofibroblast formation in a naive embryonic environment suggests molecular activation of a fundamental pathway, which if inhibited in the adult may promote wound healing post-MI, whereas the stimulation of an organised endothelial cell network is indicative of a potentially therapeutic neovascular response. Affinity chromatography and mass spectrometry studies are ongoing for target deconvolution and (novel) pathway identification. Phenotypic screens to identify active molecules, in this regard, are powerful tools towards accelerating drug-discovery(2) and the development of stem-cell-based myocardial and neovascular therapies.

41. Transcription factor heterogeneity in proepicardial and epicardium-derived cell populations during embryonic heart development

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During embryonic development, the proepicardium (PE) migrates over the myocardium to form the epicardium. Epicardial cells undergo epithelial-to-mesenchymal transition to form epicardium-derived cells (EPDCs), which invade the myocardium and differentiate into fibroblasts, smooth muscle and endothelial cells. The PE and EPDCs are comprised of heterogeneous cell populations, which differentially express transcription factors including NFATc1, WT1, and Tbx18. To what extent these factors correlate with cell fate in EPDCs is unknown. We hypothesize that the localized expression of NFATc1, WT1 and Tbx18 in PE cells and EPDCs influences EPDC cell fate. Chick embryonic day 4 (E4) PE and E7 epicardium were explanted to compare NFATc1, WT1 and Tbx18 function in PE cells and EPDCs, respectively. NFATc1, WT1 and Tbx18 are expressed in subsets of cultured PE cells and EPDCs. Therefore, these culture systems will be used to investigate cell type diversity in PE cells versus EPDCs. In a subset of cultured PE cells, NFATc1 expression colocalizes with the endothelial marker Flk1, which suggests that NFATc1 marks endothelial precursors in vitro. Tbx18 and WT1 are each expressed in subsets of EPDCs, but the fate of these cells is unknown. In addition, loss of Tbx18 function, using Tbx18 specific siRNA, significantly decreases EPDC proliferation, as detected by BrdU incorporation assay. These data suggest that NFATc1, WT1 and Tbx18 mark different cell lineages in PE and EPDC cultures, and that Tbx18 regulates EPDC proliferation. Our long-term goal is to define the molecular mechanisms that regulate PE and EPDC proliferation and cell lineage specification.

42. The Type III Transforming Growth Factor-β Receptor Regulates Proliferation and Apoptosis in Epicardial Cells

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Epicardial cells undergo transformation and form coronary vessels. Tgfbr3-/- embryos have an irregular epicardium, few coronary vessels, and die at E14.5. To explore TGFβR3 signaling, we used immortalized epicardial cell lines from E11.5 embryos. Tgfbr3+/+, Tgfbr3+/-, and Tgfbr3-/- cells form a tight epithelium. TGFβ1 or TGFβ2 (250 pM) causes transformation and induces the smooth muscle marker, SM22α in all genotypes. To determine the role of TGFβR3 in TGFβ-induced growth inhibition, dose response curves were generated for TGFβ1 or TGFβ2. All genotypes display TGFβ1-induced growth inhibition. Tgfbr3-/- cells have decreased sensitivity to TGFβ2-induced growth inhibition. Circular wounds were made in cell monolayers and the percent wound closure calculated. Tgfbr3+/+ and Tgfbr3+/- cells closed the wound by 48h. Tgfbr3-/- cells required 72h ($p<0.05$). Tgfbr3+/+ and Tgfbr3+/- cells gave similar proliferation rates when measured by BrdU, peaking at 48h (33.1% and 33.93%) and returning to basal levels (19.9% and 20.85%) by 72h. Tgfbr3-/- cells sustained a basal proliferation rate 13% lower at 48h ($p=0.001$). As a second measure of proliferation, we used the MTS assay. Tgfbr3-/- cells had a rate of proliferation 2-fold lower at 48h and 2.6 fold lower at 72h ($p<0.05$). Preliminary data indicates decreased proliferation in E13.5 epicardium in vivo. Apoptosis was determined by Apo-One Caspase 3/7 Homogenous assay. The apoptosis rate in Tgfbr3-/- cells was 1.8, 3.3 and 5.9- fold higher than Tgfbr3+/+ cells at 24, 48, 72h, respectively ($p<0.05$). In summary, TGFβR3 is not required for smooth muscle differentiation in epicardial cells, but does regulate proliferation and apoptosis.

43. The Type III Transforming Growth Factor-β Receptor to Regulates Invasion in Epicardial Cells

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A subset of epicardial cells undergoes transformation, invades the subepicardial space, and gives rise to the coronary vessels. Tgfbr3-/- embryos fail to develop coronary vessels, have an irregular epicardium, and die at E14.5. We used immortalized epicardial cell lines from E11.5 embryos to determine the invasive potential of epicardial cells in response to TGFβ stimulation in a modified Boyden chamber assay. Tgfbr3+/+, Tgfbr3+/-, and Tgfbr3-/- cells invade a collagen 1 matrix in response to 250pM TGFβ1 or TGFβ2, however, Tgfbr3+/- and Tgfbr3-/- cells show reduced invasion. Overexpression of TGFβR3 in Tgfbr3-/- cells restored invasion in response to TGFβ2. TGFβR3 lacking the cytoplasmic domain fails to support invasion. The three C-terminal amino acids – STA - are required for binding to the PDZ domain containing protein, GAIP-interacting protein, C terminus (GIPC) and are reported to be required to regulate cell migration. TGFβR3 lacking these 3 residues does not support invasion. We next asked if ALK5 is downstream of TGFβR3. Constitutively active ALK5 is sufficient to induce invasion In Tgfbr3-/- cells. Incubation of Tgfbr3+/+ cells with the ALK5 kinase inhibitor SB 431452 (2.5μM) or Calbiochem 616451 (4 μM) reduces TGFβ2-stimulated invasion by ~40%. The addition of 2.5μM SB 431452 inhibited invasion in response to TGFβ2 in Tgfbr3-/- cells overexpressing TGFβR3 demonstrating that ALK5 is downstream of TGFβR3. These data demonstrate that TGFβR3 regulates epicardial cell invasion via the GIPC-binding domain and that ALK5 acts downstream of TGFβR3.

44. Migration and differentiation of epicardial cells stimulated by TGFβ1, TGFβ2, or BMP-2 does not require the Type III Transforming Growth Factor β receptor

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Transforming Growth Factor β (TGFβ) Receptor III (TGFβR3) binds all three TGFβ ligands, inhibin, and Bone Morphogenic Protein-2 (BMP-2). Since TGFβ causes epicardial cell differentiation into smooth muscle in vitro we asked if Tgfbr3-/- epicardial cells differentiate in response to TGFβ or BMP-2. Tgfbr3+/+ and Tgfbr3-/- immortalized mouse epicardial cells (ED 11.5) were cultured in the presence or absence of ligands for 72 hours and scored by immunohistochemistry, qRT-PCR, and a LacZ reporter assay for Smooth Muscle 22 alpha (SM22α). Tgfbr3+/+ and Tgfbr3-/- cells lose epithelial character and undergo smooth muscle differentiation in response to 250 pM TGFβ1 or TGFβ2 as scored by upregulation of smooth muscle markers SM22α, smooth muscle α-actin (SMαA) or calponin. Induction of SM22α in Tgfbr3+/+ cells occurred with an EC50 of 12.5 pM. BMP-2 (5 nM) induced epithelial to mesenchymal transformation (EMT) characterized by the loss of Zonula Occludens-1 (ZO-1), but failed to induce SM22α, SMαA, or calponin. Inhibition of activin receptor-like kinase (ALK) 5 activity, a Type I TGFβ receptor that may act downstream of TGFβR3, with 2.5 μM SB431542 blocked the effects of TGFβ1 and TGFβ2, but not BMP-2. Pre- and co-incubation of cells with 5 nM BMP-2 reduced the level of smooth muscle marker induction by 250 pM TGFβ1 or TGFβ2. These data demonstrate that BMP2 induces epicardial cell motility without smooth muscle differentiation and that TGFβR3 is not required for epicardial cell responsiveness to TGFβ or BMP-2.

45. Isolation and characterisation of mouse embryonic epicardially-derived cells in engineered 3D fibrin/collagen matrices

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In the developing heart the epicardium surrounding the heart as a result of epithelial-to-mesenchymal transformation generates precursor cells that invade the myocardium and subsequently become smooth muscle, endothelial and interstitial cells. Currently only a few effector proteins and mechanisms that guide differentiation of epicardially derived cells (EPDC) are well understood. Because most processes in vivo occur within 3D tissues rather than on 2D surfaces, modern 3D tissue-based culture systems require to mimic certain aspects of in vivo-like scaffolding and reconstruction of cell adhesion, motility and cell-cell communication. The aim of this study was the development of an in vitro 3D model that would allow concomitant investigations of migration/proteolytic activity and morphogenetic abilities of EPDCs in response to matrix-bound growth factors. Eight immortalized, clonally expanded cell strands from embryonic mouse epicardium were investigated. Single cells or cell spheroids were embedded in fibrin matrices engineered with covalently bound growth factors. The spheroids of certain cell strands exhibited multicellular sprouts into fibrin and fast outward migration upon their culture with matrix-bound BMP-2, VEGF. We found that some cell strands exhibited an extraordinary proteolytic capacity, which resulted in rapid consumption of the surrounding fibrin. Molecular analysis revealed expression of numerous cell surface markers including Eph receptors, VEGFR1, CD44, CD106, CD140. Our present experiments focus on the influence of interstitial flow and matrix-bound growth factors on the spatio-temporal patterning of epicardial cells. In conclusion, we have developed an in vitro 3D cell system that allows the recapitulation of certain aspect of epicardial cell behavior.

46. Transforming Growth Factor beta ligands play essential roles in epicardium and epicardial cardiac progenitor cells during heart development

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The epicardial epithelial-mesenchymal transition (EMT) is hypothesized to generate epicardium-derived progenitor cells that differentiate into coronary smooth muscle, cardiac endothelium, cardiac fibroblasts and cardiomyocytes. The presence of Transforming Growth Factor beta2 (TGFbeta2) gene expression in the proepicardium and the gene expression of all three TGFbeta ligands in epicardium from E11.5-E12.5 onward suggest a role of TGFbeta ligands in epicardium-dependent cardiac morphogenesis. Here, we showed that TGFbeta2-deficient and not TGFbeta1- or TGFbeta3-deficient mice had reduced WT1+-epicardial progenitor cells and thinning of the ventricular myocardium at mid-gestation. TGFbeta2-deficiency resulted in reduced levels TGFbeta signaling activity (i.e., pSMAD2) in epicardium and epicardial progenitor cells. In vitro analysis using collagen gel assays indicated reduced epicardial EMT in TGFbeta2-deficient hearts. Simultaneous gene-targeted deletion of both TGFbeta2 and TGFbeta3 resulted in epicardial detachment, a phenotype that was not seen in TGFbeta2-deficient embryos. The data also identified impaired coronary vascular development in TGFbeta2-deficient embryos which was further attenuated in TGFbeta2/TGFbeta3-deficient embryos. The defect in epicardial progenitor cell differentiation in the TGFbeta2/TGFbeta3-deficient hearts was associated with a significant induction in the levels of di-phosphorylated ERK-MAP kinases. Collectively, these data indicate that TGFbeta2 is required for epicardium-dependent cardiac mophogenesis. TGFbeta3 plays specific role in epicardial progenitor cell migration into the heart by inducing epicardial attachment to the heart. These new insights into the molecular mechanisms regulating epicardial progenitor cells function will shed light on the pathogenesis of heart diseases and may help the development of novel regenerative approaches to heart failure. (Funding: R01HL92508-Conway-Doetschman; ABRC: 0901 & Schneider/Gieszl Award-Azhar)

Session A - Extracellular Matrix & Morphogenesis

47. Characterisation of the wickham mutant identifies a novel gene in cardiac looping

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Cardiac looping is a morphogenetic event that converts the symmetrical linear heart tube into an asymmetric looped heart. Defects that occur during heart development, including cardiac looping, are the underlying cause of congenital heart defects, emphasizing the importance of understanding the cellular and molecular regulators of cardiac morphogenesis. We have utilized the model organism, zebrafish, and its' amenability in forward genetic screens to identify novel genes in the process of cardiac looping. The mutant, wickham, undergoes normal formation of the linear heart tube but fails to loop, never forming an S-looped heart. Wickham hearts display normal myocardial structure and contractility suggesting a normal cardiac output at the time of looping. Furthermore, anf/nppa and tbx2b are expressed in a comparable manner between mutants and siblings, suggesting normal chamber differentiation and initiation of the myocardial AV canal boundary, respectively. In addition, no significant difference was found in myocardial cell numbers between wickham/Tg(cmlc2:dsRed) siblings and mutants ($n=6$; $p < 0.681$). In contrast with these negative findings, has2 expression was expanded in wickham mutants compared with siblings, suggesting that this defect may be endocardial in origin. Together these findings indicate that the wickham gene plays a specific role in vertebrate heart tube morphogenesis. Positional cloning of wickham identified a putative transmembrane protein with no prior ascribed function. Wickham RNA was highly expressed in a restricted manner in the mouse endocardium, providing additional evidence of an endocardial-derived defect. Based on these findings, we propose that wickham represents a novel, endocardial-expressed gene involved in cardiac morphogenesis.

48. Alk3 is required for cardiac specification and asymmetry

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During formation of the linear tube, the heart undergoes asymmetric positioning, relative to the left-right (LR) axis. LR asymmetry is established in the embryo by a Nodal-BMP signaling cascade, which is then interpreted by the cardiac field. Asymmetric positioning of the heart tube is necessary for the subsequent morphogenesis and correct looping of the heart. We have identified the zebrafish mutant, linkspoot, from a forward genetic screen, which has a symmetrical linear heart tube phenotype that is partially penetrant (approximately 1/3 of mutants). Positional cloning identified a missense mutation (L337R) in the bmp receptor, alk3a. We hypothesised that the partially penetrant phenotype resulted from compensation from the alk3a paralogue, alk3b (from duplication in the zebrafish). To test this, we screened a mutagenesis library and identified a premature stop-codon in the ligand-binding domain of alk3b. To test for redundancy, double carriers (alk3a+/-, alk3b+/-) were incrossed and the progeny phenotyped and genotyped. A gene dosage affect was observed, whereby the more copies of alk3a/b lost, the greater the penetrance of the cardiac laterality phenotype. Interestingly, double mutant embryos were unable to be scored for laterality as there was no detectable cardiac field using markers cmlc2 (cardiac myosin light chain), nkx2.5 and hand2. These phenotypes in double mutants and compound heterozygous animals indicate that while the specification of the heart field fails to occur only in the complete absence of Alk3, the morphogenesis of the heart tube is exquisitely sensitive to reductions in Alk3 dose.

49. Tenascin C may regulate recruitment of mural cells during coronary arterial development.

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Tenascin C (TNC) is a unique extracellular glycoprotein, transiently expressed at distinct sites, and thought to play important roles in tissue remodeling during embryonic development and under pathological condition in adult. Several lines of evidnce have suggested that TNC may be involved in angiogenesis in cancer stroma and tissue repair after myocardial infarction. To elucidate a role of TNC in coronary vessel development, we investigated spatiotemporal expression patterns of TNC during the establishment of proximal region of coronary arteries. Coronary precursor cells were traced using proepicardial organ quail-chick chimera. Immunohistochemistry showed that TNC was deposited in the aortic wall where endothelial invaded to form primordial coronary stems. At ED8, when a single orifice of coronary stem was completed and smooth muscle actin (SMA)-positive mural cells originated proepicardium were recruited, TNC was extensively deposited surrounding the coronary arterial wall. When nascent epicardium was cultured on dish coated with purified TNC, the cells differentiated into smooth muscle cell phenotype expressing SMA. Epicardial cells cultured without TNC showed a typical cobblestone appearance with expressing endothelial marker QH1. These results revealed that TNC plays a significant role in coronary artery development, especially recruitment of mural cells by accelerating differentiation of epicardial mesenchymal cells to SMA positive cells.

50. Type XIV collagen is required for myocardial function in vivo

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Type XIV collagen (Col14) is a fibril-associated collagen with interrupted triple helix (FACIT) required for fibrillogenesis in tissues of high mechanical stress. The purpose of this study was to define its role in cardiac function. We show that Col14 is highly expressed within the extracellular matrix of the working myocardium from late embryonic stages through adulthood. From three months of age Col14a1-/- mice exhibit significant defects in myocardial function, as determined by echocardiography. This is indicated by increased left ventricular chamber volume (and diameter) during diastole (23%, p=0.04) and systole (43%, p=0.02) in conjunction with an overall 23% and 28% decrease in ejection fraction and fraction shortening respectively. These functional studies suggest that Col14a1-/- mice have moderate ventricular dilation and significantly impaired contractility. At the molecular level, ventricles from Col14a1-/- mice express significantly reduced levels of genes associated with cell-cell and cell-matrix interactions including type VI collagen (1.9-fold, p=0.02), integrin alpha-2 (3.7-fold, p=0.03) and laminin beta-3 (1.6-fold, p=0.03). This apparent decrease in structural integrity within the ventricle of Col14a1-/- mice likely reflects the observed pathologic alteration in function. Ongoing studies include biomechanical approaches to determine the maximal and passive force of individual muscle fibers from Col14a1-/- mice, as well as electron microscopy to examine the ultrastructure of extracellular matrix assembly within the ventricles of null animals. These current studies suggest that Col14 is required for cytoskeleton integrity, and function of the working myocardium in vivo.

51. Results of a forward genetic screen to identify new genes that regulate heart morphogenesis

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Morphogenesis of the vertebrate heart is characterized by looping and leftward positioning of the heart tube. We and others have shown that, in zebrafish, this is a BMP and Nodal regulated process. During early heart morphogenesis in zebrafish, an asymmetric positioning of the heart takes place, resulting in the positioning of the future venous pole towards the left side of the body axis while the arterial pole remains at the midline. Following leftward positioning, rightward looping takes place; a repositioning of the developing atrial and ventricular chambers relative to one another occurs, bringing the chambers closer in lateral alignment. This is accompanied by ballooning of the two chambers and a constriction between the atrium and ventricle to form the atrioventricular canal. By forward genetic screening in zebrafish, putative novel genes that have important functions during cardiac positioning and looping have been identified. Embryos that are mutant for these putative genes can be divided into three classes based on their phenotypes. Class I mutants display defects in cardiac positioning and looping combined with endodermal laterality defects. Class II mutants show defective cardiac positioning and looping while the laterality of the gut is unaffected. Class III mutants display cardiac looping defects combined with a normal gut morphology. Standard genetic mapping approaches will be used to identify the genes underlying the mutant phenotypes. Genes that can be mapped to regions on the genome that do not harbour known regulators of heart morphogenesis will undergo further analysis.

52. Mechanotransduction and Erbb2 Signaling in Cardiac Trabecular Formation

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Cardiac trabeculae are highly organized sheet-like muscular structures that protrude into the ventricular chambers. Trabeculae have been suggested to enhance contractility and intraventricular conduction. However, the mechanisms that orchestrate cardiac trabecular development remain largely unknown. Here we show that mechanosensing mechanisms are required for cardiac trabecular formation. Trabeculae fail to form in silent heart (sih/ctnnt2) mutant embryos, in which the cardiomyocytes fail to contract leading to circulation failure. In addition, transplanted non-contractile cells in wildtype ventricles fail to incorporate into the trabeculae. Interestingly, myocardial cells that overexpress an activated form of RhoA, a known mediator of mechanosensitive changes, are exclusively detected in the trabeculae. Similar to silent heart, mutants for erbb2, which encodes an epidermal growth factor (EGF) receptor family member, also fail to form trabeculae. In addition, transplanted erbb2 mutant myocardial cells in wildtype ventricles do not incorporate into the trabeculae. BrdU labeling demonstrates that ErbB2 is required for myocardial proliferation during trabecular forming stages. However, lineage-tracing studies along with the presence of isolated BrdU+ trabecular cells suggest that directional migration, in addition to proliferation, contributes to trabeculation. These observations suggest that mechanotransduction and ErbB2 signaling are required for the detachment of prospective trabecular myocardial cells from the ventricular wall.

53. Obscurin promotes the targeted localization and stabilization of the dystrophin associated glycoprotein complex and participates development of the costameres, and the myotendinous and neuromuscular junctions

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Obscurin is a giant protein with multiple structural and signaling domains that plays a pivotal role in the cellular organization of skeletal and cardiac myocytes. It surrounds the myofibril at the M and Z bands and recruits other cytoskeletal and transmembrane proteins to those domains. Given obscurin's role in cellular patterning, we hypothesized that it would promote organization of specialized membrane compartments overlying the sarcomere. To test this hypothesis, we used antisense morpholino oligonucleotides to diminish obscurin A in developing zebrafish embryos and characterized the effect on specialized membrane compartments that are dependent upon dystrophin and the dystrophin-associated glycoprotein complex (DGC). In the obscurin-depleted -dystroglycan expression was not noted at theαembryos, glycosylated myotendinous junctions suggesting a specific destabilization of DGC. Ultrastructural analysis indicated that obscurin depletion resulted in reduced affinity of the myofibril for the sarcolemma, and wheat germ agglutinin staining supported a lack of costamere organization and development in the obscurin-morphant embryos. The obscurin-depleted embryos also had reduced numbers and organization of neuromuscular junctions as indicated by -bungarotoxin. The findings suggest that obscurinαimmunolabeling with participates in the organization and development of dystrophin/DGC-dependent membrane compartments. The DGC is a vital transmembrane link between the subcortical cytoskeleton and the basal lamina and has been implicated in the etiology of several muscular dystrophies. Understanding obscurin's role in the assembly and organization of these specialized membrane compartments may have important implications for the treatment of patients with a wide range of myopathies and muscular dystrophies.

54. The role of the small GTPase Cdc42 in the Drosophila heart during morphogenesis and function

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The formation of the heart during Drosophila embryogenesis requires the coordinated alignment of the myocardial and pericardial cells. One important aspect of heart morphogenesis is therefore the establishment and maintenance of cell polarity. A number of factors, including the secreted protein Slit and its receptor Robo localize in a polarized manner during and after heart alignment and further morphogenesis of the heart. It is currently not known which factors create or maintain this polarized distribution and localization of factors. From a screen for genetic interactors with the cardiac determinant tinman we identified the small GTPase Cdc42 as being required for correct adult heart function. In addition, we find that it governs cardioblast cell polarity during embryonic heart morphogenesis. Perturbing cell polarity by either loss of Cdc42 or expression of constitutively active Cdc42 disrupts heart morphogenesis in the embryo. As a consequence, multiple heart lumina are formed. We have screened for factors that influence cardiac cell polarity in a Cdc42-dependent manner, in order to understand the mechanisms of cardiac morphogenesis.

55. Structural determinants of material properties of fetal and adult porcine mitral heart valves

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The proper material properties and withstanding life long cyclic mechanical loading is essential for maintaining of normal mitral heart valve function. Material properties of heart valve must also constantly adapt to changes in cardiovascular hemodynamics during embryonic and postnatal development. Inadequate or suboptimal mechanical properties of heart valve tissues can compromise them and lead to heart failure. The main goal of this work is to estimate material properties of porcine mitral heart valve in fetal, postnatal and adult stage and identify their structural determinants. Using tensile test we have shown that material properties of porcine mitral heart valves are progressively improved and heart valve became more strengthen with increasing modulus of elasticity. Transmission electron microscopy demonstrated the increase in diameter of collagen fibrils in adult valve as compared to fetal heart valve. Collagen content based on hydroxyproline test increases 5 times as compared fetal to adult heart valve. Level of collagen cross-linking is lower in fetal heart valve then in adult heart valve based on estimation of thermal denaturation temperature by differential scanning calorimetry. Thus, structural determinants of progressive age-related changes in material properties of heart valves include increasing of collagen type 1 content, diameter of collagen fibrils and level of collagen cross-linking. These data provide necessary control and important insight on mechanisms of embryonic and postnatal valvulogenesis, pathogenesis of heart valvulopathies and they are essential in designing of functional tissue engineered heart valves.

56. Matrix engineering for the formation of artificial tissues

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Naturally derived matrices have well been established as 3D culture systems. One mayor limitation of these hydrogel materials in engineering applications is their innate biological functions regarding proteolytic susceptibility and integrin ligand presentation. We have in recent years developed completely synthetic matrices that are modularly designed and can be adapted towards specific biological applications. These materials are based on star shaped poly (ethylene glycol) PEG, a polymer known to be biologically inert. In order to obtain biological functionality the materials can be made sensitive towards proteolytic digestion and decorated with specific integrin ligand domains or covalently immobilized morphogenetic cues that might direct cell behaviour. Here we show that cells can be encapsulated inside these materials and that by variation of the matrix composition cell migration behaviour can be enabled or prohibited in vitro. As these matrices allow the covalent tethering and presentation of growth factors, they can not only be used to control distribution and migration of cells, but also provide them with instructive cues. Initial attempts have been made to control the 3D arrangement of cells, matrices and growth factors to generate tissue-like structures by means of layer by layer assembly or printing. These constructs were shown to convert over time to become tissue like constructs with defined arrangement. We believe that our novel materials platform can serve to generate tissue mimicking structures that can be used to study fundamental biological questions such as cell differentiation, tissue morphogenesis, cell-cell and cell-matrix interactions that are important during development.

57. The Novel Transmembrane Protein Frv is Essential for the Coordination of Myocardial and Endocardial Morphogenesis

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The myocardium and endocardium are closely juxtaposed throughout heart development. Myocardial and endocardial cells originate in neighboring regions of the lateral mesoderm, migrate medially in a coordinated fashion, collaborate to create concentric layers of the heart tube, and communicate during formation of the atrioventricular canal. Here, we identify a novel gene, frozen ventricle (frv), that plays an essential role in the coordination of myocardial and endocardial morphogenesis in the zebrafish embryo. Zygotic frv mutants exhibit ectopic presence of atrioventricular characteristics throughout the ventricular endocardium, indicating an important role of Frv in restricting formation of the endocardial cushions to the atrioventricular canal. Furthermore, in maternal-zygotic frv mutants, both myocardial and endocardial cells fail to move to the midline normally. Positional cloning indicates that the frv locus encodes a type II single-pass transmembrane protein. Homologs of frv are present in all examined vertebrate genomes, but nothing is known about its molecular or cellular function in any context. Although frv is expressed throughout the embryo, tissue-specific rescue experiments indicate that Frv activity is required in the myocardium to coordinate the movement of both myocardial and endocardial cells. Together, our data reveal that Frv is an essential mediator of myocardial-endocardial communication during cardiac morphogenesis.

58. Prognostic utility of physiologic assessment of extracellular matrix fiber alignment and tissue biomechanics in detection of latent aortic disease

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Aneurysmal patients with mutations in Transforming Growth Factor beta (TGFbeta) signaling (i.e., Loey-Dietz syndrome, LDS) and collagen (i.e., Ehlers Danlos syndrome, EDS) have higher incidence of sudden lethal aortic rupture than patients of Marfan syndrome (MFS) and familial thoracic aortic aneurysm (TAAD). Here, we present unique opto-biomechanical approaches that had successfully determined the susceptibility to aortic aneurysm in mouse models at the pre-aneurysm stage. Briefly, our data indicated that TGFbeta2-deficient and TGFbeta2/TGFbeta3-deficient mouse embryos exhibited widespread aortic artery dilation. Although adult TGFbeta2-haploinsufficient mice appeared normal, a pressor dose of angiotensin-2 (ANG2) for 7-days selectively induced thoracic aortic aneurysm in these mice. Our newly developed microbiaxial opto-mechanical device is capable of simultaneously and non-invasively investigating the macroscopic biomechanics (stress/strain: tissue-stiffness) and microscopic (via nonlinear multiphoton excitation) microstructural extracellular matrix (ECM) changes (collagen and elastin) in an unfixed biaxially-pressurized mouse aorta. The opto-mechanical analyses indicated that aortic walls were more compliant in TGFbeta2-haploinsufficient mice and that they were less compliant in both Fibrillin-1+/C1039 (MFS model) and ANGII-infused (TAAD model) mice as compared to wild-type mice. The alignment of collagen fibers in pressurized TGFbeta2-haploinsufficient mouse aorta remained axially oriented and that the elastin fibers moved more circumferentially as compared to the wild-type mice. Collectively, these data establish approaches capable of predicting the risk of aortic aneurysm, and indicate higher susceptibility of TGFbeta2-haploinsufficient mice to aortic aneurysm. These findings have implications in devising better strategies for advancing aortic rupture risk assessment in the susceptible pediatric aneurysmal patients. (Funding: R01HL92508-Conway-Doetschman, ABRC: 0901 & Schneider/Gieszl Award-Azhar.

Session A - Neural crest

59. Trigenic neural crest-restricted Smad7 over-expression results in congenital craniofacial and cardiovascular defects

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60. Cardiac Neural Crest Nibble Their Way to the Heart

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For abstract, see in oral presentation section page 76

61. Manipulation Of Notch Signaling In Neural Crest Cells In Vivo Results In Cardiac Outflow Tract Malformations

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The Notch signaling pathway has been implicated in cardiac congenital malformations and diseases including Alagille syndrome and aortic valve calcification. During embryogenesis, precise levels of Notch1 signaling regulate cell proliferation and differentiation in a variety of cell types. In the developing cardiac outflow tract, Notch signaling has been linked to congenital conotruncal malformations that are often attributed to defective neural crest cell contribution. While Notch signaling molecules are expressed in neural crest cells, their role is undefined. We hypothesize that Notch signaling regulates cardiac neural crest cell proliferation, migration, and differentiation thereby affecting subsequent investment into the cardiac outflow tract. RosaNotch mice were crossed with Wnt1Cre mice for over-expression of activated Notch1 in all neural crest cells and their derivatives in the developing embryo. Wnt1Cre;RosaNotch mice exhibit a reduced number of migrating cardiac neural crest cells leading to outflow tract malformations, including persistent truncus arteriosus, and lethality by embryonic day 14.5. Furthermore, Notch loss of function mice in the neural crest lineage (Wnt1Cre;Notch1fl/fl and Wnt1Cre;RBP-Jfl/fl) have decreased neural crest cell migration resulting in an array of cardiac outflow tract malformations including double outlet right ventricle and overriding aorta with ventricular septal defects and neonatal lethality. These data demonstrate that Notch signaling in neural crest cells is required for conotruncal development and that deficient Notch signaling leads to congenital cardiac malformations. Overall, these studies provide evidence that precise levels of Notch signaling are required in neural crest cells, which has critical implications in cardiac development and disease.

62. Rho kinase is required for cohesive behaviour of neural crest cells during outflow tract morphogenesis

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Neural crest cells (NCC) are essential to the development of the cardiac outflow tract. However, the factors regulating the local movements and condensation of NCC are currently poorly understood. To address this we studied the effect of the conditional expression of a dominant-negative form of Rho kinase (ROCK) in NCC, which suppressed the activity of endogenous ROCK solely in NCC resulting in severe craniofacial, innervation and cardiac defects. NCC were able to migrate efficiently in ROCKDN;Wnt1Cre embryos although many of these cells died either at the periphery or within the neural tube. Close examination of migrating NCC and post-migratory cells as they condensed in the outflow cushions, revealed that whereas wild type cells migrated as a cohesive population, ROCKDN;Wnt1Cre NCC were disorganised and tended to clump together or scatter, suggesting abnormalities in NCC cell-cell adhesion. In vitro analyses of isolated NCC confirmed that NCC migration was not impeded by ROCK deficiency, although cell-cell adhesion and gap junctional proteins involved in cell-cell communication between neighbouring NCC were all mis-localised within the cells. Furthermore, cell tracking experiments demonstrated that attenuation of ROCK signalling resulted in accelerated and more directional NCC movement leading to separation of leading edge cells. Thus ROCK appears to be essential for the behaviour of NCC by regulating their final co-ordinated movements and condensation within target tissues. ROCK acts in a number of signalling pathways and this therefore explains how disruption of distinct upstream factors can result in the same cardiac malformations.

63. Replacement of a large minority of smooth muscle cells by melanocytes in ductus arterisosus during development does not allow its proper closure and leads to postnatal death

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Melanocytes may be defined by their neural crest cell (NCC) origin and their ability to produce melanin using the key enzyme tyrosinase (Tyr). In vertebrates, these pigmented cells are classically found in the skin (classical melanocytes) but also in diverse sites: eyes, inner ear, meninges, bones and heart (non-classical melanocytes). The functions of most non-classical melanocytes remain unknown. Mice expressing an oncogenic form of β-catenin (βcat) specifically in tyrosinase-producing cells were generated using the Cre/LoxP system (Tyr::Cre/°;Δex3βcatflox/+). These mice unexpectedly died prematurely (between 8 and 18 weeks of age) from cardiac defects. They displayed a considerably dilatated left atrium and died consequently to the rupture of the left atrium with pericardial blood effusion. Moreover, most of the animals presented a thrombus in the left atrium. A series of Echo-Doppler and histological analysis was performed to determine the origin of this dilatation. We observed a high number of melanocytes in the mutant heart and the lack of closure of the ductus arteriosus (DA) at birth, thus connecting the pulmonary artery directly to the aorta. We could show that the wild-type DA is composed of two vagal NCC derivatives - smooth muscle cells (SMC) and a very small amount of melanocytes. At the opposite, mutant DA contained fewer SMC and a larger number of melanocytes. These results suggest that higher levels of β-catenin in vagal NCC has a possible effect on the fate of these cells, with larger numbers of cells differentiating into melanocytes, thus preventing the closure of the DA.

64. Cross-talk between T-box factors in cardiac outflow tract morphogenesis

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Proper cardiac outflow tract (OFT) morphogenesis is a complex process that is crucial for the formation of the aortic and pulmonary outlets. The addition of cells from two cell populations, the second heart field (SHF) and cardiac neural crest (NC) cells, to the developing heart is required for OFT development and septation. T-box transcription factor Tbx1 is expressed in the SHF and is known to be important for SHF and OFT development. Tbx2 is expressed in both SHF and cardiac NC cells, while Tbx3 is mainly expressed in cardiac NC cells. Both factors are required during OFT remodeling. However, little is known about their putative hierarchical places in gene regulatory networks and signaling pathways controlling OFT development. Here, we made 3D-reconstructions of Tbx1, Tbx2 and Tbx3 expression patterns in the SHF and NC region and found that Tbx1/Tbx2 and Tbx1/Tbx3 are largely complementary, whereas Tbx2/Tbx3 largely overlap in the SHF, NC-derived mesenchyme, ectoderm and foregut endoderm. In Tbx1-deficient mice, pharyngeal expression of Tbx2 and Tbx3 was found to be deviant, suggesting regionalized interactions. To study in vivo interactions and define the combinatorial roles, we have generated compound Tbx1;Tbx2 and Tbx2;Tbx3 mutant mice. Tbx2;Tbx3 double mutant mice display a shortened OFT, hypoplastic right ventricle and abnormal patterning of the pharyngeal arch arteries. Tbx1;Tbx2 double mutants showed arrested development from E8.75 onwards and a primitive and dysmorphic heart tube. Our data suggest that Tbx1 is required upstream of and in synergy with Tbx2 and Tbx3 for proper cardiac and pharyngeal arch development, and that interactions between Tbx1-expressing and Tbx2/3-expressing cells may play a crucial role in this process.

65. The role of AP-2α in the neural crest cells for cardiovascular development

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Congenital cardiovascular malformations (CCVM) are the most common human birth defects and are associated with multiple human syndromes. Many CCVMs affect the pharyngeal arch arteries (PAA) and the outflow tract (OFT) of the heart. During development the PAAs undergo extensive remodelling from a symmetrical structure into the highly asymmetric aortic arch arteries. Faults in the formation and rearrangement of these vessels result in severe aortic arch malformations. Neural crest cells (NCCs) play an integral role in the development of these structures, producing the smooth muscle wall of the PAAs and infiltrating the distal OFT to initiate the formation of the aorticopulmonary septum. AP-2α (Tcfap2a) is a transcription factor associated with cell proliferation. Embryos deficient in AP-2α are characterised externally by craniofacial abnormalities and ventral body wall defects, and display cardiovascular defects such as double outlet right ventricle, ventricular septal defects (VSD), interrupted aortic arch (IAA) and anomalous right subclavian artery (A-RSA). As this phenotype partly affects neural crest-derived structures, we wanted to investigate the role of AP-2α within NCC for cardiovascular development. To achieve this, we are selectively deleting Tcfap2a from NCC using the cre-LoxP system. Preliminary data shows that the NCC-specific ablation of Tcfap2a, in mice on a congenic C57Bl/6 background, causes VSD, A-RSA and transposition of the great arteries, in addition to exencephaly. Understanding the requirement for AP-2α in the NCCs may provide insight into the genetic pathways involved in cardiovascular development, which are affected in human syndromes showing CCVM.

66. Twist-Family Member Interactions Regulate Cardiac Neural Crest Morphogenesis

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Members of the Twist-family of basic helix-loop-helix (bHLH) transcription factors, including Twist1, Hand1 and Hand2, have been shown to perform essential functions during cardiac neural crest cell (cNCC) development. Systemic ablation of Twist1 causes cNCC-associated abnormalities in the dorsal neural tube, pharyngeal arches, and a subpopulation of OFT cNCCs expressing Hand1 and Hand2. The use of tissue-specific gene ablation models has deepened our understanding of the roles these factors play in cNCC migration, adhesion, patterning and differentiation and, ultimately, congenital heart disease. Deletion of Hand2 specifically in the neural crest has been shown to result in OFT defects including aortic arch mispatterning and double outlet right ventricle. Conditional inactivation of Twist1 in the premigratory neural crest demonstrates that Twist1 function is necessary for aortico-pulmonarty septation and for proper cNCC contribution to the OFT cushions. A hallmark of Twist-family members is their ability to form homo- and heteromeric molecular complexes with unique transcriptional properties. Relative gene dosage of Twist1 and Hand2 within the developing limb is critical, as genetic disruption of the balance of these two factors causes polydactyly. Current studies employing systemic and NCC-specific inactivation of these factors will define cooperative and/or antagonistic Twist1 and Hand2 functions within cNCCs. Together, these data will broaden out understanding of the mechanisms by which bHLH factors molecularly interact and transcriptionally regulate cNCC contribution to the developing OFT, and define, in terms of gene transcription and both cellular and genetic interaction, the role of the bHLH code in OFT development.

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

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Cardiac neural crest cells (CNCC) in mice and chick give rise to the smooth muscle of the aortic arch arteries and form the aorticopulmonary septum. Zebrafish develop aortic arch arteries and have conserved arterial pole development. Two previous studies in zebrafish reported the origin of CNCC, but the maps were very different. In addition, these studies found CNCC throughout the heart that co-localized with a striated muscle marker, an unexpected cell fate for CNCC. Although these studies were done carefully, neither took a direct approach of tracing genetically labeled neural crest cells. Using the neural crest transgenic reporter sox10:egfp line, we used uncageable red dye to map the origin and destination of green fluorescent neural crest cells as they began to migrate in the three cranial streams and adjacent to somite one. Our results showed that neural crest cells from the first cranial stream and somite one do not populate any structures near the heart. Neural crest cells from the second and third cranial streams come in close proximity to the developing heart fields in early migration at 16 hours post-fertilization (hpf) and are heavily concentrated in the caudal branchial arches at 48 hpf; but no cells were ever found to co-localize with a myocardial marker. These preliminary studies suggest CNCC originates from the second and third cranial streams and that CNCC do not differentiate into myocardial cells prior to 48 hpf. We have not yet ruled out whether CNCC are added later.

68. The neural crest-enriched microRNA, miR-452, regulates epithelial-mesenchymal interactions in cranial neural crest derivatives

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Neural crest cells (NCCs) are a subset of multipotent, migratory stem cells that populate myriad tissues during development and are important for craniofacial and cardiac morphogenesis. Although microRNAs (miRNAs) have emerged as important regulators of development and disease, little is known about their role in NCC development. Here, we show that loss of miRNA biogenesis by NCC-specific disruption of Dicer results in embryos lacking craniofacial cartilaginous structures, cardiac outflow tract septation, thymus, and dorsal root ganglia. Dicer mutant embryos had reduced expression of Dlx2, a transcriptional regulator of pharyngeal arch development, in the first pharyngeal arch (PA1). miR-452 was enriched in NCCs, was sufficient to rescue Dlx2 expression in Dicer mutant pharyngeal arches, and regulated a signaling cascade involving Wnt5a, Shh, and Fgf8 in PA1 that converged on Dlx2 regulation. Correspondingly, knock-down of miR-452 in vivo decreased Dlx2 expression in the mandibular component of PA1, leading to minor craniofacial defects. These results suggest that post-transcriptional regulation by miRNAs is required for differentiation of NCC-derived tissues and that miR-452 is involved in epithelial-mesenchymal signaling in the pharyngeal arch.

Session A - Valve Development

69. MiR-23 is essential to restrict endothelial-to-mesenchymal transition during cardiac valve formation.

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For abstract, see in oral presentation section page 67

70. Cardiac valve malformations: new insights from Pdlim7, an unexpected suspect in heart development

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For abstract, see in oral presentation section page 68

71. Krox20 is required during valve remodeling and maturation

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For abstract, see in oral presentation section page 69

72. The Metalloprotease ADAMTS5 Plays A Critical Role In ECM Stratification And Cusp Sculpting During Semilunar Valve Maturation

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For abstract, see in oral presentation section page 70

73. Induction of a developmental gene program in pediatric and adult aortic valve disease

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For abstract, see in oral presentation section page 71

74. Reduced Sox9 function promotes heart valve calcification in vivo

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For abstract, see in oral presentation section page 72

75. TGF-β3 and MMP3 contribute to the pathogenesis of a myxomatous mitral valve in canines.

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Myxomatous degeneration of the mitral valve is observed in primary mitral valve prolapse (MVP) or marfan syndrome (MFS) in humans. The myxomatous changes are characterized by an accumulation of proteoglycans and a disruption of the collagenous and elastin matrix architecture. The studies of a murine model of MVP and MFS suggested that either enhanced matrix metalloproteinases (MMP) or transforming growth factor-beta (TGF-β) signaling may be related to the pathogenesis of myxomatous mitral valve. It has been reported that canine myxomatous mitral valve disease is similar to MVP in humans, and could be used as the models of human MVP. We compared TGF-βs and MMPs expressions in the normal mitral versus myxomatous valves in canines. Expressions of TGF-β3, TGF-β receptor II, smooth muscle α-actin (αSMA) and matrix metalloproteinase 3 (MMP3) were only observed in myxomatous valves. In vitro culture system, TGF-β3 induced significantly increase levels of αSMA protein and proteoglycans. TGF-β3 activation and secretory rates were stimulated by MMP3 treatment. Total amount of TGF-β3 in valve interstitial cells (ICs) significantly decreased by angiotensin-converting enzyme inhibitor (ACEI) and angiotensin II receptor blocker (ARB). Our findings suggest that increased TGF-β3 and MMP3 contribute to the pathogenesis of a myxomatous degeneration. In addition, it is possible that ACEI and ARB could effectively block the degeneration of mitral valves.

76. A Novel Role for Tie1 in Late Gestational Semilunar Valve Remodeling

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Tie1 is an endothelial specific receptor tyrosine kinase required for developing stable vasculature, endothelial cell survival and quiescence. Global knockouts of Tie1 result in embryonic lethality at about E13.5 due to vascular defects. No ligand for Tie1 has been identified, and downstream targets of receptor activation remain unknown. To circumvent early embryonic lethality and decipher the role of Tie1 in latter stages of valve development, our laboratory has developed a floxed Tie1 allele and a Cre transgenic line (NFATc-P2Cre) that is specific for the endocardium overlying the endocardial cushions and not in mesenchyme derived from EMT or other endothelial populations. Having documented efficient Cre mediated excision of Tie1 in the endocardium, we observed that aortic valves lacking Tie1 are hypertrophic. Mutant mice have an aortic valve area up to 2X greater than WT littermates from E14.5 through adulthood, though overall cell number remains unchanged. Interesting, no effect on pulmonary valve development was observed. Approximately half of the mutant mice die between E16.5 and P0. No alterations in endocardial or mesenchymal cell proliferation or apoptosis were detected, nor were abnormalities in endocardial cell morphology observed. However, extracellular matrix production of the valvular interstitium was clearly perturbed and an increase of proteoglycans and glycosoaminoglycans were visualized by Movat's pentachrome stain. Immunostaining for periostin, a mesenchymal cell specific pericelluar matrix protein, suggested a delay in matrix remodeling. Because Cre mediated gene deletion was restricted to the endocardium and not detected in any mesenchymal cells, these data suggest that active endocardial to mesenchymal signaling, at least partially mediated by Tie1, is required for normal remodeling of the valves in the late gestation embryo.

77. FGF-BMP signaling axis in the valve development

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Congenital valve defects are common birth defects, but the underlying mechanism is poorly understood. Both OFT septum and OFT valve are derived from OFT cushion. However, how OFT cushion gives raise to these two distinct structure is unclear. In this report, we demonstrated that muscularization regulates the formation of OFT valve. By combination of multiple mouse genetic manipulations, cell lineage tracing, ex vivo heart culture, and molecular biology approaches, we showed fibroblast growth factor (FGF) signaling regulates valve formation through BMP4. Uncoupling these two signaling axes by double ablation of Fgfr1 and Fgfr2, single ablation of Frs2α or Bmp4, disrupted muscularization and resulted in enlarged OFT valves.

78. Snai1 is required for MMP15 expression during endothelial to mesenchymal transformation and endocardial cushion formation

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Mature heart valve structures are derived from endocardial cushions (ECs) that form during embryonic development as a result of endothelial to mesenchymal transformation (EMT). This EMT process involves several phenotypic changes in endothelial cells including loss of cell-cell interactions and the acquisition to transform and migrate through matrix-rich tissue. The transcription factor Snai1 has previously been shown to be required for EMT in many developmental systems through the repression of cell adhesion molecules, however its direct function in EC EMT has not been examined. In this study, we observed Snai1 expression in endothelial and newly transformed mesenchyme cells of the ECs prior to and during EMT processes. To determine its function, Snai1 was overexpressed in avian EC explants in vitro, and conditionally knocked down in vivo using murine models. We show that increased Snai1 function is sufficient to promote EMT processes as indicated by significantly increased cell migration and transformation. In support, mice with reduced Snai1 function in endothelial-derived cells exhibit a 39% (p=0.04) reduction in the number of mesenchymal cells within the ECs compared to controls. At the molecular level, this phenotype is associated with a 4.8-fold decrease (p=0.02) in MMP15 expression. Interestingly, EC explants treated with recombinant active MMP15, show increased cell migration with no change in cell transformation. This study shows that Snai1 is required for EC EMT, and identifies a new mechanism of Snai1-mediated function for cell migration during this developmental process.

79. The Role of microRNA-21 in Zebrafish Cardiac Valve Formation.

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Recent research has revealed that atrioventricular valves (AVV) formation requires mechanical forces such as heart beating and blood flow. AVVs are lost when heart beat is disturbed by knocking down cardiac troponin T (Tnnt2) or treating embryos with BDM, cardiac ATPase inhibitor. The aim of our current research is to reveal what mediates mechanical forces with valvulogenesis. We hypothesized microRNAs (miRs) are one of the possible candidates from two reasons. First, some miRs are involved in cardiac defects and dysfunctions. Secondly, some miRs are up (down) regulated during cardiac hypertrophy implying their mechano-dependent expression. Hence, we searched miRs expressed in zebrafish hearts and found z-miR-21 expression in cardiac valves. MiR-21 is specifically expressed in endocardium of developing AVV. Moreover, miR-21 expression is downregulated by blocking heart beat suggesting miR-21 expression depends on mechanical force. To confirm whether the mechanical force is involved in miR-21 expression, we conducted promoter assay on miR-21 genomic region. MiR-21 promoter contains serum response factor (SRF) and E26 transforming sequence1 (ETS1) binding sites, which are highly conserved among various vertebrates. RhoA activates miR-21 expression through SRF site, and ETS1 also increased promoter activity. Previous reports suggest these signaling have responsiveness for mechanical force. Knock down by antisense morpholino oligo against miR-21 invites AVV formation defects. Furthermore, epithelial to mesenchymal transition (EMT) during valve formation fails to occur in miR-21 morphants. Taken together, miR-21 expression triggered by mechanical stress contributes to AVV formation. We'll show updated datum on the function of miR-21 promoter responding to mechanical stress.

80. Loss of the Type III Transforming Growth Factor-β Receptor is associated with enlarged AV cushions

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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. The Type III TGFβ receptor (TGFβR3) binds all TGFβ ligands and several BMP ligands. Gain- and loss-of-function experiments have demonstrated a requirement for TGFβR3 in TGFβ-stimulated EMT. Tgfbr3-/- mice have double outlet right ventricle, ventricular septal defects, and a thinned myocardium. Tgfbr3-/- embryos die at E14.5 due to failed coronary vessel development. Tgfbr3-/- embryos at stage E13.5 have enlarged atrioventricular (AV) cushion volumes compared with Tgfbr3+/+ and Tgfbr3+/- littermates. Given this finding, we asked if AV cushions were hypercellular or simply enlarged due to an increase in, or dysregulation of, the extracellular matrix. We used ImageJ software to calculate the AV cushion areas of 10 serial sections from E13.5 Tgfbr3+/+ and Tgfbr3-/- littermates and counted the total number of cells. No significant difference was seen in the number of cells per square micron in the Tgfbr3-/- embryos (0.0034 ± 0.0013) and Tgfbr3+/+ embryos (0.0031 ± 0.0011) (n=3, p=0.06). These data suggest that the enlarged cushion volume is accompanied by an increase in total cell number. We next examined the rate of proliferation in AV cushions in E13.5 littermates. Tgfbr3-/- AV cushions showed decreased proliferation (19.7% ± 9.8%) when compared with Tgfbr3+/+ embryos (30.3% ± 9.8%) (n=2, p=0.02). Taken together, these data show that E13.5 Tgfbr3-/- embryos, when compared to Tgfbr3+/+ embryos, have increased AV cushion volume, equivalent cell density in the cushions, and a decreased rate of cell proliferation.

81. Shear stress induced endoMT: the role of endothelial cilia.

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Primary cilia are mechanosensors for fluid shear stress and sensitize endothelial cells (EC) for shear. A flow related distribution of ciliated endothelial cells in the embryonic cardiovascular system has previously been described, with ciliated endothelial cells restricted to the areas of low shear in the embryonic heart. In the adult, primary cilia are located in areas of disturbed flow, a distribution reminiscent of atherosclerosis predisposition sites. In vitro cilia can be induced by flow disturbances, suggesting a role for primary cilia in endothelial activation and dysfunction. We studied the functional effect of cilia on the shear response of embryonic endothelial cells. To this aid, transgenic mouse embryonic endothelial cells (Tg737orpk/orpk) which are not able to build functional cilia were exposed to shear stress in a dedicated bioreactor. The morphological and functional response to flow was assessed by analyzing a set of shear and EndoMT markers. The response of Tg737orpk/orpk cells was compared to that of wild type ciliated embryonic EC. Wild type EC showed a normal response to shear accompanied by induction of the transcription factors Klf2 and Klf4, whilst Tg737orpk/orpk EC underwent EndoMT to gain a fibroblast like phenotype. This shear stress induced transition could be prevented by either interfering with the Tgf-β or shear induced Klf signaling. In the current study we provide evidence for the role of cilia in defining a functional response of endothelial cells to fluid flow.

82. Position of coronary artery ostia in conotruncal defects

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Abnormal coronary ostia are frequently associated with conotruncal defects particularly with common arterial trunk (CAT). Recent study in mouse has shown that Tbx1 controls outflow tract development and coronary artery patterning, with subaortic coronary-permissive and subpulmonary coronary-refractory myocardial domains. In order to determine if abnormal outflow tract development influences coronary ostia position, we rewieved heart specimens with conotruncal defects : 46 CAT, 29 tetralogy of Fallot with pulmonary atresia (TOF&PA), 15 tetralogy of Fallot (TOF), 11 double-outlet right ventricle with subaortic ventricular septal defect (DORV) and 17 normal anatomy (control). Position of the coronary ostia over the aortic or truncal circumference was measured in degrees as the direction from the middle of the valvar orifice. The left coronary ostium was more posterior in conotruncal defects vs control (mean angle : control=0°, TOF=31°, TOF&PA=47°, DORV=44°, CAT=65°, $p<0.005$) especially in CAT vs other conotruncal defects ($p<0.05$). The right coronary ostium was more anterior in TOF, TOF&PA and DORV vs control (mean angle : control=213°, TOF=242°, TOF&PA=245°, DORV=271°, $p<0.05$) especially in DORV vs TOF and TOF&PA ($p<0.05$), but not in CAT (195°). The anterior intercoronary angle, which corresponds to the pulmonary identity domain, was similar in TOF, TOF&PA, DORV and control (133°-162°) but significantly larger in CAT (229°, $p<0.0001$). Anomalies of outflow tract rotation responsible for conotruncal defects significantly affect the position of the coronary ostia. The marked difference between CAT and other conotruncal defects could reflect the impact of associated septation anomaly on the width of the subpulmonary coronary-refractory domain.

83. Anomalies of coronary artery ostia in common arterial trunk.

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Tbx1 controls outflow tract development and coronary artery patterning in mouse, with subaortic coronary-permissive and subpulmonary coronary-refractory myocardial domains. Our hypothesis is that the potential “pulmonary identity” of the common arterial trunk (CAT) influences coronary ostia anomalies. Thus, we reviewed 46 heart specimens with CAT and 17 with normal anatomy (control). Position of the coronary ostia over the truncal circumference was measured in degrees as the direction from the middle of the valve orifice. CAT types were: 28 type I, 8 type II, 3 type III, 7 type IV. The truncal valve was bicuspid in 2, tricuspid in 36, quadricuspid in 7. Overall, 87% cases had malformations of the coronary ostia size, shape, location relative to the commissures or sinotubular junction. Left coronary ostium had abnormal shape or size in 33/46, right coronary ostium in 20/46 ($p<0.01$). Left coronary ostium was located above a commissure in 17% vs 2% for right coronary ostium ($p<0.05$) and above the sinotubular junction in 28% vs 10% ($p=0.053$). Left coronary ostium was more posterior than in control (65° vs 0°, $p<0.0001$), right coronary ostium was located similar to control (195° vs 213°). The anterior intercoronary angle, reflecting pulmonary identity domain, was 229° in CAT vs 147° in control ($p<0.0001$). Left coronary ostium in CAT is more frequently abnormal than right coronary ostium. This might be due to the obligatory dorsal connection of left coronary ostium because of the large coronary-refractory subpulmonary domain in the ventral left part of the CAT.

84. Notch and Bmp2 signaling integration during valve formation

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Despite the prevalence of cardiac valve disease, little is known about the molecular circuitry regulating heart valve morphogenesis. We find that Notch and Bmp2 act coordinately to establish a valve-forming field between two chamber developmental domains. Patterning occurs via the activation of epithelial-to-mesenchymal-transition (EMT) in non-chamber endocardium. Mice with constitutive endocardial Notch1 expression activate a mesenchymal gene program in ventricles (Snail1-2, Twist2, Tgfß2) and a partial ectopic EMT in vitro that becomes invasive upon BMP2 treatment. Inhibition of Snail1, Tgfβ2 or Notch1 reduces BMP2-induced ventricular transformation and invasion, while BMP2 treatment inhibits endothelial Gsk3β, stabilizing Snail1 and promoting invasiveness. Integration of Notch and Bmp2 signals is demonstrated by attenuated Notch1 transcription and signaling after Bmp2 myocardial deletion, while Notch1 myocardial activation extends Hey1 expression to non-chamber myocardium, represses Bmp2 and impairs EMT. We propose that the interplay between myocardial-Bmp2 and endocardial-Notch1 restricts EMT to prospective valve territory.

85. PLD1 is required for endocardial cell transformation in vitro

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A pivotal early step in valvulogenesis occurs when a subpopulation of endocardial cells overlaying the pro-valvular cardiac cushions undergo an epithelial to mesenchymal transformation (EMT). These mesenchymal cells seed the cardiac cushions, eventually remodeling the extracellular matrix and contributing to the valves and septa of the adult heart. Phospholipase D (PLD) catalyses the hydrolysis of phosphatidylcholine into phosphatidic acid (PA) and choline. PA acts as a lipid second messenger to regulate cell processes including adhesion, migration, cytoskeleton organization, and cancer progression. Although PLD1 and PLD2 are linked to EMT in cancer cells and migration in adult endothelial cells, the role of PLD in endothelial cell EMT is unknown. To address the role of PLD in endocardial cell EMT, atrioventricular cushions were explanted from Hamburger-Hamilton Stage 14 chicken embryos onto collagen 1 gels and incubated with small molecule inhibitors specific for PLD1 (VU0155069, 5μM), PLD2 (VU0285655-1, 5μM), or a pan PLD inhibitor (VU0155056, 5μM). The number of cells entering the gel was decreased by 87.5% ±3.10% (n=3) with PLD1 inhibition, 18.2% ±12.6% (n=3), with PLD2 inhibition, and 72.8% ±3.90% (n=3) with pan inhibition. Similar results were obtained with 500nM of inhibitor. To confirm the involvement of PLD, incubation with 1-butanol (0.6%) resulted in a 75.6% ±3.86% (n=3) decrease in cell invasion while 3-butanol (0.6%) had no effect when compared to vehicle alone. These data are the first to implicate PLD in endocardial cell EMT in vitro and suggest PLD1 as a candidate downstream effector for growth factors that regulate valvulogenesis.

86. Arsenic Exposure Perturbs Epithelial-Mesenchymal Cell Transition (EMT) and Gene Expression In a Collagen Gel Assay

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Arsenic is a naturally occurring metalloid and environmental contaminant. Arsenic exposure in drinking water is reported to cause cancer of the liver, kidneys, lung, bladder and skin as well as birth defects including neural tube, facial and vasculogenic defects. The early embryonic period most sensitive to arsenic includes a variety of cellular processes. One key cellular process is epithelial-mesenchymal transition (EMT) where epithelial sheets develop into three-dimensional structures. An embryonic prototype of EMT is found in the atrioventricular (AV) canal of the developing heart, where endothelia differentiate to form heart valves. Effects of arsenic on this cellular process were examined by collagen gel invasion assay (EMT assay) using explanted AV canals from chicken embryo hearts. AV canals treated with 12.5-500 ppb arsenic, showed a loss of mesenchyme at 12.5 ppb and mesenchyme formation was completely inhibited at 500 ppb. Altered gene expression in arsenic-treated explants was investigated by microarray analysis. Genes whose expression was altered consistently at exposure levels of 10, 25, 100 ppb were identified and results showed that 25 ppb in vitro was particularly effective. 382 genes were significantly altered at this exposure level. Cytoscape analysis of the microarray data using the chicken interactome identified 4 clusters of altered genes based upon published relationships and pathways. This analysis identified cytoskeleton and cell adhesion-related genes whose disruption is consistent with an altered ability to undergo EMT. These studies show that EMT is sensitive to arsenic, and that an interactome-based approach can be useful in identifying targets.

87. Developmental Basis of Myxomatous Mitral Valve Disease

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Myxoid cardiac valves is a common feature in a heterogeneous group of disorders that includes Marfan syndrome and isolated valvular diseases. Mitral valve prolapse is the most common outcome of these and remains one of the most common indications for valvular surgery. While the etiology of the disease is unknown, recent genetic studies have demonstrated that an X-linked form of familial cardiac valvular dystrophy can be attributed to mutations in the Filamin-A gene. Since these inheritable mutations are present from conception, we hypothesize that filamin-A mutations present at the time of valve morphogenesis lead to dysfunction that progresses postnatally to clinically relevant disease. Therefore, by carefully evaluating genetic factors (such as filamin-A) that play a substantial role in MVP, we can elucidate relevant developmental pathways that contribute to its pathogenesis. In order to understand how developmental expression of a mutant protein can lead to valve disease, the spatio-temporal distribution of filamin-A during cardiac morphogenesis was characterized. We demonstrate that filamin-A is robustly expressed in non-myocyte cells throughout cardiac morphogenesis including epicardial and endocardial cells, and mesenchymal cells derived by EMT from these two epithelia, as well as mesenchyme of neural crest origin. In postnatal hearts, expression of filamin-A is significantly decreased in the atrioventricular and outflow tract valve leaflets and their suspensory apparatus. Mechanistic studies have suggested that filamin-A can be post-translationally modified by a post-EMT, valve-specific enzyme and in doing so, promotes the compaction and organization of the extracellular matrix. These studies provide mechanistic insight into how cytoskeletal changes, mediated by enzymatic post-translational modifications can drive maturation of the valve.

88. Bmp-2 And Tgfβ2 Shared Pathways Regulate Endocardial Cell Epithelial-Mesenchymal Transformation

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Valvular heart disease is a major cause of mortality and morbidity. Revealing the cellular processes and molecules that regulate valve formation and remodeling is required to develop effective therapies. A key step in valve formation during heart development is epithelial-mesenchymal transformation (EMT) of a subpopulation of endocardial cells in the atrioventricular cushion (AVC). The Type III Transforming Growth Factor β Receptor (TGFβR3) regulates AVC endocardial cell EMT in vitro and mesenchymal cell differentiation in vivo. Relatively little is known concerning the signaling mechanisms downstream of TGFβR3. Here we use endocardial cell EMT in vitro to determine the role of two well characterized downstream TGFβ signaling pathways in TGFβR3-mediated endocardial cell EMT. Targeting of Smad4, the common mediator Smad, demonstrated that Smad signaling is required for EMT in the AVC and TGFβR3-mediated EMT stimulated by TGFβ2 or BMP-2. Although we show that Smads1, 2, 3, and 5 are required for AVC EMT, overexpression of Smad1 or Smad3 is not sufficient to induce EMT. Consistent with the activation of the Par6/Smurf1/RhoA pathway downstream of TGFβR3, targeting ALK5, Par6, or Smurf1 significantly inhibited EMT in response to either TGFβ2 or BMP-2. The demonstrated requirement for ALK5 activity, Par6, and Smurf1 for TGFβR3-mediated endocardial cell EMT is consistent with the documented role of this pathway in the dissolution of tight junctions after RhoA degradation. Taken together, our data demonstrate that TGFβR3-mediated endocardial cell EMT stimulated by either TGFβ2 or BMP-2 requires Smad4 and activation of the Par6/Smurf1/RhoA pathway.

89. Periostin mediates Transforming Growth Factor beta2 function in valve development

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Transforming Growth Factor beta (TGFbeta) play important role in cardiac cushion epithelial-mesenchymal transition (EMT) and cushion remodeling, and Periostin (POSTN) which is a fasciclin-containing adhesive glycoprotein facilitates the differentiation of cells that have undergone EMT during cushion remodeling and maturation. In situ hybridization revealed that TGFbeta2 and TGFbeta3 genes were expressed in complementary fashion to POSTN gene in developing cardiac valves. Both TGFbeta2-deficient and POSTN-deficient mice exhibited cushion remodeling defects. Here, we present in vitro and in vivo data which are consistent with the notion that an elevated TGFbeta signaling which induces POSTN is involved in valve thickening in TGFbeta2-deficient mice. Immunohistochemical analysis showed that TGFbeta2-deficient, and not TGFbeta1- or TGFbeta3-deficient, valvular fibroblasts had increased levels of POSTN in cardiac valves. In vitro studies using mouse embryonic fibroblasts (MEFs) showed that POSTN gene expression was similarly increased in TGFbeta2-deficient MEFs. Further experiments showed that elevated POSTN gene expression seen in TGFbeta2-deficient MEFs was significantly reduced by TGFbeta signaling inhibitor (SB-431542). The data also revealed the existence of a feedback mechanism. Briefly, TGFbeta2-deficient MEFs induced TGFbeta1 gene expression when challenged with the exogenous TGFbeta1. In addition, TGFbeta signaling effectors pSMAD2 and pSMAD1/5 increased sharply in response to TGFbeta treatment in TGFbeta2-deficient MEFs as compared to wild-type MEFs. Finally, these findings were consistent with the elevated levels of TGFbeta1 in cardiac cushions of TGFbeta2-deficient mice. In conclusion, these data provide evidence that Periostin is a downstream target gene of TGFbeta signaling and that it mediates TGFbeta2 function during valvulogenesis. (Funding-R01HL92508-Conway-Doetschman.

90. Origin, Development, and Fate of the Dorsal Mesenchymal Protrusion

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Proper septation and segmentation of the 4-chambered heart is critically dependent on the development of the atrioventricular mesenchymal complex (AVMC). The AVMC is formed by the fusion of several mesenchymal tissues in the developing heart, including the major atrioventricular (AV) cushions, the mesenchymal cap on the leading edge of the primary atrial septum, and the dorsal mesenchymal protrusion (DMP). Immunohistochemical studies, in combination with lineage tracing using Tie2-cre and Mef2C-AHF-cre mouse models, have demonstrated that while the AV cushions and “cap” are endocardially-derived, the DMP is an anterior (or second) heart field-derived tissue. Our recent studies on the Ts16 mouse and Crtl1 knock-out mouse, and studies by others using different mouse models, demonstrate that abnormal development of the DMP is associated with the pathogenesis of atrioventricular septal defect (AVSD; Snarr et al., 2007b; Wirrig et al., 2007; Goddeeris et al., 2008). AVSD is a congenital heart malformation characterized by an ostium primum atrial septal defect, and in many cases ventricular septal defect, a common AV valve, and a persistent AV canal. AVSDs are found in approximately 5% of the general population of all patients suffering from congenital heart disease and in 20-25% of individuals with Down Syndrome. Our lab is interested in elucidating the role of the DMP in valvuloseptal morphogenesis. Here we will present new data that will further understanding of the origin, development and fate of the DMP.

91. Elastin Haploinsufficiency is Associated with Progressive Aortic Valve Malformation, Latent Valve Disease and Decreased TGF-beta Signaling in a Mouse Model

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Divisions of Cardiology (1), Molecular Cardiovascular Biology (2), Bioinformatics (3), Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, and Department of Cell Biology and Physiology (4), Washington University, St Louis, Missouri

Elastin is an extracellular matrix (ECM) component of heart valves. Based on findings in a patient with elastin haploinsufficiency and valve disease, we hypothesized that elastin insufficient mice (Eln+/-) would manifest viable heart valve disease. Heterozygous mutant mice were analyzed at neonatal, juvenile, adult and aged adult stages; homozygous mutants were analyzed at the neonatal stage only. Valve function was evaluated in vivo using echocardiography (VisualSonics). Juvenile mice demonstrated normal valve function, but valve disease was identified in 17% adult and 70% aged adult Eln+/- mice (predominantly aortic insufficiency). Histochemical analysis demonstrated normal morphology and ECM organization at the neonatal stage, attenuation of the annulus including fragmented elastin infiltration of the annulus at the juvenile stage, thin elongated cusps and ECM disorganization at the adult stage, and overgrowth of the annulus with proteoglycan accumulation at the aged adult stage. Ultrastructure analysis in Eln+/- mice demonstrated loss of ECM stratification, increased proteoglycans, and annular elastin fragmentation. Neonatal Eln+/- mice demonstrated increased valve interstitial cell (VIC) proliferation (p-HistoneH3), and neonatal juvenile and adult mice showed VIC activation (SMA, SMemb). Interestingly, TGF-beta signaling (p-Smad2) was decreased in neonatal Eln-/- mice; there was a modest but not statistically significant decrease in Eln+/- mice. However, gene expression analysis (Affymetrix) identified a significantly decreased TGF-beta mediated fibrogenesis signaling pathway in juvenile Eln+/- mice. These findings identify the Eln+/- mouse as a model of latent valve disease, implicate decreased TGF-beta as an underlying mechanism of maladaptive ECM remodeling, and establish a role for elastin in valve pathogenesis.

FRIDAY 21 MAY - SESSION B

Time: 13.00 - 14.30 hours
Location: Marble Hall
1st floor and basement

Session Topics

Cardiac Progenitor Cells	[posters 92 - 115]
Epigenetics	[posters 116 - 119]
Heart Fields	[posters 120 - 133]
Miscellaneous	[posters 134 - 135]
Transcription Regulation	[posters 136 - 168]

Session B - Cardiac Progenitor Cells

92. Endothelial progenitors contribute to the heart endocardium and play key roles in cardiac development

Michal Milgrom-Hoffman*(1), Napoleone Ferrara(3), Elazar Zelzer(2) and Eldad Tzahor(1)
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For abstract, see in oral presentation section page 37

93. Identification of micro RNAs involved in cardiac specification in mESCs

Alexandre Colas*, Wesley McKeithan, Mark Mercola
Muscle Development and Regeneration Program, Sanford|Burnham Medical Research Institute, La Jolla, CA, USA
For abstract, see in oral presentation section page 38

94. Regulation of cardiac progenitor cells during heart development

Francesca Rochais* and Robert G. Kelly
Developmental Biology Institute of Marseilles-Luminy, CNRS UMR 6216-Université de la Méditerranée, 13288 Marseille, France.
For abstract, see in oral presentation section page 39

95. Hematopoietic cell contribution to embryonic mouse valve development and adult valve homeostasis

Zoltan Hajdu* (1), Roger R Markwald (1), Christopher C Drake (1), Imre Olah (2), Richard P Visconti (1)
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For abstract, see in oral presentation section page 40

96. Intrinsic Fluorescence: Non-invasive Signatures of Embryonic Stem Cells and the Implications for Multiphoton Flow Cytometry

Jayne M. Squirrell* (1,2), David G. Buschke (2,1), Amber Mael (3), Kevin W. Eliceiri (1,2), Gary E. Lyons (4,1), Nirupama Shevde (3), Timothy J. Kamp (5,1), and Brenda M. Ogle (2,1)
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Advances in cell research and cell therapies, such as repair of cardiac tissue following infarction, depend on technologies that accurately and non-invasively assess cell state, both as single cells and as 3D entities. Defining intrinsic biomarkers that characterize stem cell state advances this goal by reducing the need for extrinsic labels. Several pieces of evidence suggest that pluripotent cells are metabolically distinct from differentiated cells. Since autofluorescent metabolites can be noninvasively detected by advanced optical methods, we reasoned that these endogenous fluorophores might serve as unique, intrinsic indicators of differentiation state. Indeed, we have identified changes in the autofluorescent properties of embryonic stem cells during differentiation, utilizing multiphoton laser scanning microscopy (MPLSM), with its ability to probe deep within multicellular aggregates, and fluorescence lifetime imaging (FLIM), for detecting changes in the fluorescence microenvironment. Using a wavelength to excite the metabolic intermediate nicotinamide adenine dinucleotide (NADH), we found that the fluorescence lifetime of NADH decreases during the initiation of differentiation, in both mouse and human embryonic stem cells. Furthermore, cardiomyocytes developed from human embryonic stem cells exhibit longer fluorescence lifetimes than non-beating cells. These differences could be exploited by screening and sorting technologies. We are currently combining these observations with a modular, stage-mounted multiphoton flow cytometry (MPFC) system that could ultimately sort cellular aggregates, such as embryoid bodies or engineered constructs, based on their specific endogenous fluorescence signatures.

97. Endogenous Retinoic Acid Regulates Cardiac Progenitor Differentiation

Song-Chang Lin (1), Pascal Dollé (2), Lucile Ryckebusch (3), Michela Noseda (4), Stéphane Zaffran(3), Michael D. Schneider (4), and Karen Niederreither* (1)
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Retinoic acid (RA) has several established functions during cardiac development, including actions in the fetal epicardium required for myocardial growth. An open question is if retinoid effects are limited to growth factor stimulation pathway(s) or if additional actions on uncommitted progenitor/stem populations might drive cardiac differentiation. Here we present the first report of dual effects of RA-deficiency on cardiac growth factor signaling and progenitor/stem biology using the mouse retinaldehyde dehydrogenase 2 (Raldh2) knockout model. While early heart defects in Raldh2-/- embryos result from second heart field abnormalities, it is unclear whether this role is transient or whether RA has sustained effects on cardiac progenitors. To address this, we employed transient maternal RA supplementation to overcome early Raldh2-/- lethality. By E11.5-14.5 Raldh2-/- hearts exhibit reduced venticular compact layer outgrowth and defective coronary artery development. While reductions in -catenin expressionβFgf2 and target pERK levels occur, no alterations in Wnt/ are observed. Cell proliferation is increased in compact zone myocardium, while cardiomyocyte differentiation is reduced, alterations suggesting progenitor defects. We report that the fetal heart contains a reservoir of stem/progenitor cells, which can be isolated by their ability to efflux a fluorescent dye, and that retinoid signaling acts on this fetal cardiac side population (SP). Raldh2-/- hearts display increased SP cell numbers, with selective increases in expression of cardiac progenitor cell markers and reduced differentiation marker levels. Hence, while lack of RA signaling increases cardiac SP numbers, simultaneous reductions in Fgf signaling reduce cardiomyocyte differentiation, possibly accounting for long-term defects in myocardial growth.

98. Notch Post-translationally Regulates β-Catenin Protein in Stem Cells and Human Cancer Cells

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Human stem cells and cancer cells are characterized by self-renewing properties, but can be induced to differentiate with appropriate cues. Notch and Wnt signaling often play critical yet antagonistic roles in such cell fate decisions, suggesting the presence of reciprocal regulation. Wnt signals promote self-renewal of stem and cancer cells by inhibiting the APC-dependent destruction complex, thereby post-translationally stabilizing β-Catenin protein. Human mutations in APC result in excess β-Catenin and are linked to development of colorectal cancer. Here, we describe a second, APC-independent mechanism for post-translational regulation of β-Catenin that involves the receptor/transcription factor, Notch. In vitro, Notch negatively regulated β-Catenin protein and Wnt activity in embryonic stem cells, lineage-specific progenitor cells as well as human APC-mutant colorectal cancer cells. In vivo, expression of Notch in cardiac progenitor cells countered the excess proliferation induced by mutant β-Catenin protein resistant to APC-mediated degradation. This study reveals a previously unrecognized function of Notch in negatively regulating active β-Catenin protein levels, independent of the APC-degradation complex, in stem and cancer cells.

99. Characterization of RCSD1 during heart development

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Duboraya has recently been shown to be important for zebrafish left-right asymmetry via non-canonical Wnt signalling. In human, the Duboraya homologue CapZIP (CapZ interacting protein) is a key player in modulating the actin cytoskeleton during stress. In this study, we have identified the Duboraya homologue in Xenopus laevis and mouse, named RCSD1. In both organisms, several splice variants could be detected by RT-PCR and sequencing. RCSD1 is localized in the cytoplasm as well as in the nucleus in cell lines. Nuclear import of RCSD1 depends on phosphorylation by JNK. In mouse, RCSD1 is expressed in the developing heart as well as in neural tissue and paraxial mesoderm. In Xenopus, the first heart-specific expression can be detected at stage 20 in the cardiac progenitor cell population. Loss of function studies in murine ES cells indicate a requirement for RCSD1 during cardiac development. Similar, loss of function analysis in Xenopus using a specific antisense morpholino oligonucleotide revealed that RCSD1 is required for normal heart development but not left-right asymmetry or cilia formation. Our data identify RCSD1 as a novel key molecule for early cardiac development.

100. A new model to determine cardiomyocyte progenitor cell differentiation and function

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Purpose: The origin and potential of cardiomyocyte progenitor cells (CMPCs) in the adult heart remains unclear. The aim of this project is to study if CMPCs, recruited from the circulation or resident in the heart, contribute to myocardial repair after damage. Methods: Mouse models are generated that can be used to visualize differentiation of progenitor cells into a specific cell type, whilst at the same time allowing controlled removal of these cells. A β-actin-promoter drives the expression of a flox-eGFP-stop-cassette, nitroreductase (NTR) and IRES-LacZ (ActENIL). NTR itself is harmless, but converts the pro-drug CB1954 into an apoptosis-inducing agent. By placing Cre-recombinase under control of an early cardiac enhancer element, the expression of LacZ and NTR is targeted to CMPCs. Results: Mouse embryonic stem cells (mESC), stably expressing ActENIL, were transfected with CMV-Cre, leading to LacZ and NTR expression. Addition of CB1954 resulted in death of LacZ+ cells. To test cell-type specificity, Tie-2-Cre was transfected into ActENIL-mESC to target endothelial cells. After embryoid body formation, addition of CB1954 resulted in abrogated vessel formation. Chimeric mice were produced using ActENIL-mESC. Additionally, to target developing cardiomyocytes, nkx-enhancer element (NEE)-Cre and tamoxifen inducible NEE-mER-Cre-mER mice were generated. We are currently validating these mouse lines for the presence of eGFP, and Cre mediated expression of LacZ and NTR. Furthermore, we are determining the time-frame of the NEE-promoter activity during embryonic development. Ultimately, these newly developed mouse lines will provide a system to observe the contribution of CMPCs to cardiac repair after MI.

101. Isolation and characterization of multipotent human NKX2-5 cardiovascular progenitors

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Cardiac progenitor cells give rise to the major cellular components of the heart. Understanding how they form and what they can become has important implications for elucidating the mechanisms underlying (abnormal) heart development. In addition, the ability to isolate these cells from pluripotent stem cells allows characterization of their properties and possibly expansion in culture, a crucial step for advancing cardiac translational medicine. Functional analysis of these cells has been hampered by the paucity of lineage specific markers and inefficient, undefined differentiation procedures. Here we describe the targeted modification of two independent human embryonic stem cell (hESC) lines in which EGFP was inserted into the locus of NKX2-5, one of the earliest transcription factors expressed in heart development. EGFP fluorescence driven by the endogenous NKX2-5 promoter faithfully reported cardiovascular lineage commitment of differentiating hESC under defined culture conditions. The early NKX2-5 positive cell population showed multilineage differentiation potential to cardiomyocytes, endothelial and vascular smooth muscle cells. NKX2.5 overlapped only partially with previously reported selection markers for the cardiovascular lineage. These data are compatible with expectations from heart development and identify a progenitor population that can give rise to the three major cell types of the heart. Taken together, these experiments demonstrate the utility of hESC for analyzing the previously inaccessible events of human cardiac lineage specification.

102. The origin and migration of secondary heart field progenitors

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In vertebrates, the progenitor cells that will become the heart are among the first cell lineages to be established and to gastrulate through the primitive streak. In the chicken embryo cardiac progenitors that form the myocardium have been mapped to the anterior two thirds of the primitive streak. Lineage analyses have demonstrated the presence of primary and secondary heart field progenitor cells, which contribute to different parts of the heart. The 'two heart field' analogy describes two waves of cardiac progenitor cell migration into the heart, which occur at different times. The cardiac progenitors that primarily contribute to the outflow tract of the heart are termed the anterior or secondary heart field cells (AHF/SHF). In the chicken embryo, AHF/SHF progenitors have been located in the splanchnic mesoderm of HH stage 8 embryos. However it is not known where these cells originate from in the earlier embryo, how they migrate or what key factors control their migration. Heart malformations in human are generally due to defects in heart tissue formed by the secondary heart field, thus studies investigating the processes that regulate secondary heart field development are essential for understanding and potential treatment of heart disease. We have used DiI and DiO labeling and immunohistochemistry techniques to map the origins of AHF/SHF progenitor cells along the primitive streak in chicken embryos. Live video microscopy has shown that the migration of these cells mirrors that of other cardiac progenitors, however, their route is slightly wider. Current experiments investigate whether ASF/SHF progenitors respond to extrinsic cues, with a focus on the potential role of Wnt11B in cardiac progenitor cell movements.

103. Identification of human embryonic stem cell-derived early cardiomyocytes based on CAG promoter driven GFP expression

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Human embryonic stem cell (hESC)-derived cardiomyocytes and cardiac progenitors are promising models for pharmaceutical studies and may provide new possibilities in therapeutic approaches. We have applied the transposon based Sleeping Beauty (SB) system to introduce a GFP reporter construct into hESCs. The SB system is a non-viral gene delivery method that has been used to introduce a selectable marker for transgene expressing undifferentiated hESCs. The gene-modified hESCs expressed all the accepted stem cell marker proteins. We induced spontaneous differentiation of the transfected hESCs into cardiomyocytes by using the embryoid body (EB) system. EB formation and the subsequent spontaneous differentiation were not altered by the reporter gene delivery. The intensity of GFP expression was found to be exceptionally high in hESC derived cardiomyocytes when the reporter gene was driven by the CAG promoter (Orbán et al., Stem Cells 2009). We found that a positive correlation between the CAG promoter-driven high GFP expression and the expression of cardiomyocyte-specific genes also existed in the case of early cardiomyocytes. The cell population with extra high GFP expression also expressed the activated leukocyte cell adhesion molecule (ALCAM) at a high level. During spontaneous differentiation, ALCAM expression reached a maximum level on the cell surface when the "GFP extra high" population appeared, prior to the appearance of spontaneously contracting areas. mRNA levels of ALCAM and of early cardiac specific transcription factors were also expressed at a maximum level prior to early cardiomyocyte formation. Our results suggest that these markers may be applicable to identify cardiac progenitors.

104. Development of the Preseptation Cardiac Arterial Pole is Conserved in Zebrafish

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The cardiac arterial pole consists of the myocardium surrounding the semilunar valve and the smooth muscle just distal to it in the vertebrate heart. These tissues are derived from the secondary heart field (SHF), which adds to the cranial heart tube to form the arterial pole before septation in animals with divided circulation. Regardless of septation, the morphology of the myocardial-smooth muscle junction has been preserved throughout vertebrate evolution. We hypothesize that the SHF is an evolutionarily conserved developmental field that contributes the arterial pole of the zebrafish heart. Using tracing techniques, we mapped cells in the medial portion of the heart field and show that these cells contribute to the arterial pole. After early heart tube formation in zebrafish, cells in the ventral pharynx were labeled and followed into the arterial pole of the heart tube. Additionally, we used cardiac precursor markers to identify this population. Because arterial pole development in higher vertebrates is dependent on tbx1 and hedgehog signaling for proper development we examined tbx1 and smoothened (hedgehog receptor) mutant zebrafish. Both myocardial and smooth muscle components of the arterial pole are disrupted in these mutants. Using DiI-labeling of the SHF, we show that these progenitors do not move into the heart in these mutants. This data suggests that morphology, transcriptional regulation and signaling pathways important in arterial pole development are conserved.

105. Characterization of myocardial progenitor cells derived from mouse embryonic stem cells

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Mouse ESCs provide a potentially unlimited supply of cells that are capable of differentiating into all of the cell types necessary for myocardial repair. We hypothesize that mESC-derived cells that are committed to a cardiac mesodermal fate will be able to exhibit significant myocardial repair in a mouse model of myocardial infarction. Since our focus is to characterize a ventricle-specific precardiac lineage, we are using two transgenic mESC lines that express either ECFP under the control of the MLC2v promoter or EmGFP under the control of the Irx4 promoter. Both of these genetic markers are ventricular-specific. At 3 and 4 days of differentiation as embryoid bodies in hanging drops, the cells were isolated by FACS either for either Flk1+/CD31- surface markers (2V cell line) or EmGFP expression (Irx4 line). These purified cell populations were expanded on feeder layers of mouse fibroblasts (STO). We are analyzing the cell-surface protein markers of these cells in vitro, and testing the ability of these cells to restore function to damaged mouse myocardium in vivo.

106. Developmental role for voltage-gated calcium channel beta subunit CACNB2 in cardiomyocyte number and adhesion.

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Voltage-gated calcium channels (VGCCs) are oligomeric complexes composed of pore-forming CACNA subunits and several auxiliary proteins. Auxiliary CACNB subunits regulate VGCC electrophysiology and chaperone CACNA subunits to the cell membrane. In humans, mutations in CACNA subunits are associated with congenital cardiac arrhythmia, but the developmental functions of the CACNB subunits are poorly understood. To determine the contributions of CACNB2 to cardiac development, we depleted zebrafish embryos of CACNB2 transcripts using morpholinos. Morphants showed no difference in fgf8 expression, suggesting that adequate numbers of cardiomyocytes were initially specified. However, later markers of cardiac progenitors in the heart field (vhmc and cmlc2), were expressed in reduced domains, suggesting fewer specified cells were present just prior to formation of the heart tube. Currently we are investigating whether altered proliferation or survival may account for the difference. Despite overt normal formation of the heart tube, cell proliferation at later stages did not fully compensate for reduced cardiomyocyte number; heart tubes in morphants contained 30% fewer cardiomyocytes at 48 hpf. Looping was reduced and cells in the outer curvature failed to undergo cell shape changes normally associated with this area of the heart. The adhesion among cardiomyocytes appeared weaker, and morphant heart tubes fragmented more easily than controls when placed under modest pressure. Thus, CANCB2 is important for assuring correct number and adhesion of cardiac progenitors; these roles might or might not be related to VGCC activity. The CACNBs, as MAGUK-family proteins, may interact with multiple protein partners via their SH3 or guanylate kinase domains.

107. Nkx Genes Are Essential for Maintenance of Ventricular Identity

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Establishment of the specific characteristics of each embryonic cardiac chamber is crucial for development of a fully functional adult heart. While recent studies in mouse highlight cooperative interactions of Nkx2-5 during ventricular formation, we have yet to understand its precise function in determining chamber-specific identity. Here, we show that nkx genes are necessary to sustain molecular attributes exclusive to the ventricle. The zebrafish genome contains two NKX2-5 homologs, nkx2.5 and nkx2.7. We have recently isolated a mutant allele of nkx2.5, a nonsense mutation that truncates the protein within the homeodomain. This mutation disrupts cardiac morphogenesis, creating diminutive ventricular and bulbous atrial chambers. Investigation of underlying cellular mechanisms reveals that the initial number of differentiated cardiomyocytes in nkx2.5 mutants is indistinguishable from that in wild-type embryos. As cardiac looping proceeds, nkx2.5 mutants first exhibit a severe collapse in the number of ventricular cardiomyocytes and an excess of atrial cardiomyocytes. To uncover shared roles of nkx genes, we employed anti-nkx2.7 morpholinos in nkx2.5 mutants and thereby reduced ventricular cell number to zero and obliterated the ventricle morphologically. In these embryos, expression of ventricular markers fades as development proceeds and is replaced by markers of atrial identity. Genetic fate map experiments are underway to confirm that ventricular cardiomyocytes transform into atrial cardiomyocytes in the absence of nkx gene function. Together, our data suggest a pivotal role for nkx genes in maintaining ventricular identity and offer a basis for investigation of therapeutic implications for patients with NKX2-5 mutations.

108. Deciphering the cellular and transcriptional hierarchy acting during the earliest step of multipotent cardiovascular progenitor specification.

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During embryonic development and embryonic stem cell (ESC) differentiation, the different cell types of cardiovascular lineage arise from differentiation of two types of multipotent cardiovascular progenitors (MCPs). It is still unclear whether these two sources of MCPs arise from the differentiation of a common progenitor and what are the molecular mechanisms that allow the specification and differentiation of these primitive MCPs. We and others have recently shown that Mesp1 acts as a master regulator of cardiovascular differentiation during ESC differentiation. Here, we generated an ESC lines expressing GFP under the regulatory region of Mesp1. We showed that Mesp1 expressing cells represent the earliest source of MCPs that are common for the primary and second heart field during ESC differentiation. By transcriptionally profiling these earliest MCPs, we uncovered different transcription factors that are co-expressed together with Mesp1 during MCPs specification. Using gain and loss of functions in ESC, we showed that the co-expression of Mesp1 with these newly identified transcription factors allowed the specification of the early cardiovascular progenitors, whereas Mesp1 and its downstream target genes are required to promote the functional maturation of MCP toward the cardiac and vascular lineages. Our study provides important insights into the cellular hierarchy that operate during cardiovascular differentiation and uncover novel regulatory mechanisms that govern intrinsically cardiovascular progenitor specification and lineage differentiation from ESCs.

109. Niche Microenvironments as Blueprint for Tissue Engineering Applications

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Stem cell-derived differentiated cells and tissues have the potential to revolutionize the field of regenerative medicine; however, transforming the promise of stem cells into therapeutically relevant treatments will require new approaches that utilize microenvironmental conditions, which are conducive to regeneration. Here we aimed to address the current limitations in our understanding of the basic biology of cardiovascular progenitor cells (CPCs). We determined the nature of the CPC niche in developing hearts and studied its role in regulating CPC self-renewal and differentiation. Using routine histology and immunohistochemistry, we identified specific extracellular matrix (ECM) molecules surrounding the CPCs in their native microenvironment in first-trimester human hearts. To explore the specific role of these ECMs, we exposed pluripotent embryonic stem (ES) cells and ES cell-derived Flk1-expressing progenitor cells that possess cardiovascular differentiation potential to collagen types I (ColI) and IV (ColIV), laminin as well as fibronectin. Utilizing flow cytometry (FACS) and gene expression analysis, we showed that exposure to ColIV as well as laminin significantly enhanced the differentiation of ES cells into Flk1-expressing CPCs when compared to cells cultured on either ColI or fibronectin. In order to be able to investigate the impact of the cardiovascular niche on CPCs in vitro as close to reality as possible, we employed electrospinning technologies to engineer three-dimensional (3D) niche substrates that served as cell culture inserts. We performed FACS analyses and found that three-dimensionality promoted cardiovascular differentiation significantly when compared to 2D control cultures. Our results will impact future approaches to regenerative cell therapy.

110. The origin of endothelial and hematopoietic cells: cell lineage analysis.

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In the early 20th century, Florence Sabin observed close spatial and temporal associations between endothelial and hematopoietic cells in avian species. These two cell types associate in the yolk-sac to give rise to blood islands, structures long recognized as the first site for blood cell emergence during mammalian and avian embryogenesis. The haemangioblast model was proposed, in which endothelial and hematopoietic lineages arise from a common precursor. Recent molecular and differentiation potential studies suggest the haemangioblast would be a mesodermal precursor established at the primitive streak before ingressing into the yolk-sac; however, these hypotheses have not been addressed in vivo. We established a mouse two-reporter genetic clonal analysis method and explored in vivo the relationship at the origin of hematopoietic and endothelial lineages. Using time- and frequency-controlled genetic induction of random-labelled cells, we have analyzed a large collection of different developmental stage embryos with clones induced pre-gastrulation. The distribution of labelled cells in blood islands at E8,5 indicates complete segregation of blood and endothelial lineages, suggesting a model in which these two lineages derive from independent epiblast populations. Blood and endothelial cell specification therefore does not involve transit through a haemangioblast precursor. The observed frequency of blood clones is 4-times that of endothelial clones, suggesting that at the time of clone induction there are 4-times more blood progenitors than endothelial progenitors. Nonetheless, the presence of massive blood cell clones in considerable numbers of embryos suggests that primitive blood cells derive from a small number of epiblast cells.

111. Opposing effects of Wif1 and Frzb on cardiomyogenesis

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During chicken cardiac development the proepicardium (PE) forms the epicardium (Epi), which contributes to several non-myocardial lineages within the heart. In contrast to Epi-explant cultures, PE explants can differentiate into a cardiomyocyte phenotype. By temporal microarray expression profiles of PE-explant cultures and maturing Epi cells, we identified genes specifically associated with differentiation towards either of these lineages and genes that are associated with the Epi-lineage restriction. We found a central role for Wnt signaling in the determination of the different cell lineages. Immunofluorescent staining after recombinant-protein incubation in PE-explant cultures indicated that the early upregulated Wnt inhibitory factor-1 (Wif1), but not the late upregulated Wnt antagonist frizzled-related protein (Frzb), stimulates cardiomyocyte differentiation. Concordingly, in the mouse pluripotent embryogenic carcinoma cell line p19cl6, early and late Wif1 exposure enhances and attenuates differentiation, respectively, while Frzb was shown to attenuate differentiation. In ovo exposure of the HH12 chicken embryonic heart to Wif1 increases the Tbx18-positive cardiac progenitor pool, confirming the stimulatory role of Wif1 on early cardiomyogenesis. These data indicate distinct regulatory roles for Wif1 and Frzb in cardiomyocyte differentiation different from those reported for other Wnt-signaling antagonists in embryonic stem cell models for cardiomyogenesis.

112. Endocardial/Myocardial Progenitor Cell Interactions during Zebrafish Heart Tube Formation

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In vertebrates, the heart is the first organ to develop asymmetrically. Recent work in zebrafish has begun to elucidate the link between L/R signaling via a Nodal-BMP signaling cascade and tissue morphogenetic events involved in cardiogenesis. While these factors have been mainly implicated in cell fate specification, newly emerging evidence suggests that Nodal and Bmp signaling regulates cell behaviors including cell migration and epithelial tissue morphogenesis. High-resolution time-lapse analyses reveal that dynamic cellular rearrangements of endocardial and myocardial cells occur during the initial stages of cardiac tube formation. Whereas cardiomyocytes within lateral positions of the heart field display mesenchymal-like squamous cell shapes and are highly motile, cardiomycytes in medial positions acquire cuboidal-to-columnar epithelial shapes. Highly motile endocardial cells are in close association with cardiomyocytes throughout heart tube formation. Modulation of Nodal-BMP signaling affects endo-/myocardial cell migration. Disruption of L/R asymmetric Nodal or BMP expression causes randomized myocardial tissue involution or inhibition of heart tube formation altogether. In functional studies, we show that BMP and Nodal signaling are essential in controlling tissue polarization and cardiac morphogenesis. To further elucidate the control of endo-/myocardial behaviors by Nodal-Bmp signaling, we have performed micro-array experiments comparing endo-/myocardial expression of wild-type, Nodal/BMP signaling-deficient, or BMP overexpressing embryos using the Agilent 44K zebrafish array. These genome-wide analyses provide a first glimpse at the potential target genes that mediate the responses of endo-/myocardial cells to Nodal-BMP signaling during cardiogenesis.

113. Three-dimensional computer imaging of adrenergic cell-derived myocardium in the adult mouse heart reveals predominantly left-sided distribution.

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To identify adrenergic derived cells in the adult mouse heart, we performed genetic fate-mapping experiments in which Cre-recombinase gene was inserted into the phenylethanolamine-n-methyltransferase locus then cross mated with the homozygous Rosa26 reporter mouse strain. This mating resulted in offspring that produced βGAL in cells of an adrenergic lineage. XGAL+ staining revealed adrenergic-derived cells throughout the adult mouse heart, however, the distribution of XGAL+ cells was predominately in the left atrium (LA) and ventricle (LV) of the heart. The XGAL+ staining in the left heart accounted for 89% of the total XGAL+ staining. The LV free wall showed intermittent staining, extending from the apex to the base of the heart, and included heavy staining of the anterior papillary muscle, along its perimeter. The right atrium (RA) and ventricle (RV) collectively showed significantly less XGAL+ staining when compared to the LA and LV (P=0.001). In addition, we examined the three-dimensional (3-D) distribution of XGAL+ cells using computer-aided reconstruction of our photomicrographs. This representation confirmed our results showing that adrenergic derived cells primarily populate the left heart. This 3-D representation is the first look at the structural architecture and localization of adrenergic derived cells within the adult mouse myocardium. Together these results demonstrate that adrenergic-derived myocardial cells contribute primarily to left heart development and may play a significant role in patterning, structural assembly, and left heart function. This novel finding could lead to developments in understanding the role of transiently adrenergic cells in the dynamic structural and functional development of the heart.

114. Induced Pluripotent Stem Cell-Derived Cardiomyocytes Exhibit Limited Gene Expression Variance and Survive after Intramyocardial Transplantation

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Purpose: To investigate 1) how much variability exists between differentiated lineages from independent induced pluripotent stem cell (iPSC) lines and, specifically, how similar iPSC-derived cardiomyocytes are to embryonic stem cell-derived cardiomyocytes and 2) if iPSCs could replace ESCs in regenerative therapy for myocardial infarction. Methods: In order to facilitate isolation of comparable cardiomyocytes and progenitor populations, we generated mouse iPSCs in which expression of Nkx2-5, an early cardiac transcription factor, is marked by transgenic GFP. GFP+ cells were selected by FACS and their gene expression was compared to GFP+ cells from 2 ESC lines containing the same reporter, using microarrays. Differentiated cells were injected in the hearts of NOD-SCID mice after coronary artery ligation, and identified by epifluorescence and immunofluorescence (n=17). Results: The Nkx2-5-GFP iPSCs formed cardiomyocytes in vivo (in chimeric embryos and teratomas) and in vitro by several induction protocols. Isolation of the iPSC-derived Nkx2-5-GFP+ cardiac progenitor pool revealed striking similarity in gene expression between independent lines (R=0.99). Only 38 annotated genes out of over 28,000 transcripts were altered greater than 2-fold compared with the ESC-derived GFP+ cells. Upon injection in the infarcted mouse heart, unselected differentiated iPSCs formed teratomas but selected Nkx2-5-GFP+ iPSC-derived cells formed cardiomyocytes only. Conclusions: Our findings suggest that despite the variability of gene expression in iPSC lines, the variance narrows significantly in specific iPSC-derived cardiac progenitors, which can be isolated and used for transplantation without generation of unwanted cell types.

115. Myocardin regulates the cardiac-smooth muscle bipotency of Isl1/SLN atrial progenitors in cardiac inflow tract

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The heart chambers and the great vessels have distinct developmental origins, yet form anatomical and functional continuity at the arterial and venous poles of the heart tube to establish and maintain the dynamism of the central circulatory system. Elucidating the mechanisms of the heart-vessel junction formation is a key to understanding the pathogenesis of the congenital and adult diseases affecting the outflow and inflow tracts. We and others have shown that early cardiac progenitors display cardiac-smooth muscle bipotency. However, it is unclear how long these cardiac progenitors maintain their bipotency and how their plasticity impacts heart-vessel boundary formation during cardiogenesis. Here we report the identification of Isl1/SLN atrial progenitors in the venous pole of the heart tube, their smooth muscle differentiation capability, and its myocardin-dependent mechanism. (1) Newly generated atrial-specific Sarcolipin

(SLN)-Cre knockin mice enabled us to identify Isl1/SLN atrial progenitors, the late subset of cardiac progenitors in the cardiac inflow tract at E10.5-13.5. (2) Isl1/SLN atrial progenitors showed cardiac-smooth muscle bipotency on cardiac mesenchymal feeder layer. (3) The muscular wall of the cardiac inflow tract consists of two layers; the inner smooth muscle layer and the outer myocardial sleeve. The atrial progenitors contributed to both cardiac and smooth muscle cells in vivo based on the lineage tracing analysis. (4) Overexpression of Myocardin in the atrial progenitors enhanced smooth muscle differentiation in vitro. (5) Myocardin-null ES cells show little contribution to the smooth muscle layer in the cardiac inflow tract but preferential contribution to the atrial myocardium in ES aggregation chimera analysis, suggesting the requirement of Myocardin in the smooth muscle differentiation of the atrial progenitors. These data indicate that the bipotency of the late cardiac progenitors is regulated by Myocardin, and suggest a possible role of the cardiac progenitor plasticity during cardiovascular morphogenesis.

Session B - Epigenetics

116. Epigenetic control of cardiac gene expression in development and disease

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Cardiac hypertrophy is characterized by transcriptional reprogramming and fetal gene activation, which correlate with cardiac performance and clinical outcome. Adult cardiomyocytes in mice are post-mitotic and express mainly α-myosin heavy chain (α-MHC), whereas embryonic cardiomyocytes are proliferative and express primarily β-MHC. Adult hearts under stress develop hypertrophy, accompanied by a shift from α-MHC to fetal β-MHC, leading to contractile dysfunction and heart failure. Mechanisms bridging the developmental and pathological gene expression are not well understood. We show that Brg1, a core ATPase component of the BAF chromatin-remodeling complex, plays critical roles in regulating gene expression, tissue growth and differentiation in embryonic hearts and adult hearts under stress. In embryos, Brg1 promotes myocardial proliferation by maintaining BMP10 and suppressing a CDK inhibitor, p57kip2. In parallel, Brg1/BAF preserves fetal cardiac differentiation by interacting with HDACs and PARP1 to transcriptionally repress α-MHC and activate β-MHC. In adults, Brg1 expression is turned off in cardiomyocytes. It is reactivated by cardiac stresses and complexes with its embryonic partners, HDACs and PARP1, to induce a pathological shift from α- to β-MHC. Preventing Brg1 re-expression decreases hypertrophy, and reverses the pathological MHC switch. Our studies uncover a role of Brg1 in maintaining cardiomyocytes in an embryonic state, and an epigenetic mechanism by which three chromatin-modifying factors, Brg1/BAF, HDACs and PARP1, cooperate to control developmental and pathological gene expressions. Furthermore, Brg1 is activated in certain patients with hypertrophic cardiomyopathy. Its level correlates with disease severity and MHC changes, suggesting a role of Brg1 in human hypertrophic heart disease.

117. Jmjd6 regulates the splicing of sFlt1 and modulates angiogenesis

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Proteins containing a Jumonji C (JmjC) domain have a central role in the epigenetic control of gene expression. This enzyme family can either act as protein hydroxylases or histone demethylases as well as by regulating splicing. The hydroxylation reaction catalyzed by jumonji proteins is depended on oxygen. Therefore, we examined the function of Jmjd6 in angiogenesis. RNAi mediated inhibition of Jmjd6 lead to a significant reduction of angiogenesis in endothelial cells (EC) in vitro (tube formation: 46±18% reduction; spheroid formation: 74±9% reduction). Moreover, silencing of Jmjd6 was associated with impaired migration (43±1% reduction). These findings were confirmed in matrigel plug assay in vivo. Jmjd6+/- mice exhibit 68±9% less FITC-lectin perfused vessels compared to wild type mice. To further elucidate the function of Jmjd6, an exon-array was performed. A dysregulation in several differential spliced gene variants including soluble Flt1 were detected. sFlt1 is an alternative splice product of the VEGF-receptor 1 (Flt1) that acts as anti-angiogenic trap for VEGF and PLGF. These findings were validated by using specific taqman probes and by measuring sFlt1 protein in the supernatant (+1045±316pg/ml). In addition, RNA pull-down experiments revealed that the Jmjd6 regulated splicing factor U2AF65 endogenously binds to sFlt1 mRNA. Exogenous added VEGF or PLGF or a specific antibody against sFlt1 significantly reduced the anti-angiogenic effect of Jmjd6 knockdown ($p<0.05$). In conclusion, these results show that Jmjd6 has an essential role in the regulation of angiogenesis by enhancing the splicing of Flt1 mRNA leading to increased levels of the anti-angiogenic short form sFlt1.

118. Imprinting mechanisms and function of Ddc, a candidate developmental gene in heart

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Dopa Decarboxylase (Ddc) is an enzyme that plays a fundamental role in the biosynthesis of catecholamine neurotransmitters and serotonin. A short form transcriptional variant of Ddc called Ddc_exon1a, which originates from an alternative promoter at exon 1a, is highly expressed in the trabecular cardiomyocytes during development of pre-natal heart and is progressively silenced during post natal development. Ddc_exon1a has recently been shown by our group to be epigenetically regulated via genomic imprinting in mouse heart in a tightly regulated tissue-specific and transcriptional variant-specific manner. Ddc and Ddc_exon1a show bi-allelic expression in all other tissues. There is growing evidence that DDC may also be imprinted in human. We aim to show, by analysing Ddc+/- and Ddc-/+ knockout mice, how ablation of Ddc_exon1a affects heart-specific development. We also will look at downstream target genes using microarray analysis. We predict that the knockout may affect compaction of the myocardium during mid-gestation, which could lead to cardiomyopathies in adults. It has recently been shown that Ddc imprinting is controlled by epigenetic mechanisms via a differentially methylated region located in CGI2 of adjacent Grb10, another gene imprinted in development, CGI2 contains a putative CTCF binding site. We present our current findings examining the role of the insulator protein CTCF using ChIP analysis to assay binding this protein at this gene locus and a potential role in imprinting.

119. Imprinting of dopadecarboxylase is conserved between mouse and human trabecular cardiomyocytes

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Epigenetic mechanisms such as DNA methylation and histone modifications regulate gene expression and influence myocardial morphogenesis. Imprinting is a parent-of-origin specific mode of gene regulation governed by epigenetic mechanisms and we have shown that an isoform of Dopadecarboxylase (Ddc), an enzyme involved in the biosynthesis of catecholamine neurotransmitters and serotonin is imprinted. Ddc_exon1a is expressed tissue-specifically and exclusively from the paternal allele in murine cardiomyocytes. The temporal and spatial expression characteristics of Ddc_exon1a suggest a link between imprinting and cardiac development. Other catecholamine/monoamine pathway enzymes have established roles in cardiogenesis and serotonin signalling pathways, which are dependent upon Ddc activity, play pivotal roles in myocardial proliferation and differentiation and in cardiac function in adults. We are examining the role of Ddc_exon1a to understand what epigenetic mechanisms are controlling this gene in the mouse heart. We are performing comparative studies in the human heart and have evidence that the expression of the gene is conserved and that DDC_EXON1A is also imprinted in human cardiomyocytes.

Session B - Heart Fields

120. Tbx5 is required in the Second Heart Field for Atrioventricular Septation

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For abstract, see in oral presentation section page 73

121. Cooperative roles of *Tbx1* and *Tbx3* during early outflow tract development

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For abstract, see in oral presentation section page 74

122. Clonal analysis reveals common lineage relationships between head muscles and second heart field derivatives in the mouse embryo

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Head muscle progenitors in pharyngeal mesoderm are present in close proximity to cells of the second heart field, and show overlapping patterns of gene expression. However it is not clear whether a single progenitor cell gives rise to both heart and head muscles. We now show that this is the case, using a retrospective clonal analysis in which an nlaacZ, converted to functional nlacZ after a rare intragenic recombination event, is targeted to the αc-actin gene, expressed in all developing skeletal and cardiac muscle. We distinguish two branchiomeric head muscle lineages, both of which also contribute to myocardium. The first gives rise to the temporalis and masseter muscles of the jaw and also to extraocular muscles, thus demonstrating a contribution from pharyngeal as well as prechordal mesoderm to this anterior muscle group. Unexpectedly, this first lineage, which contributes to first branchial arch derivatives, also contributes to myocardium of the right ventricle. The second lineage gives rise to muscles of facial expression, regarded as second branchial arch derivatives. It also contributes to myocardium at the base of the arteries, that derives from the outflow tract, at the arterial pole of the heart. Further sub-lineages distinguish myocardium at the base of the aorta or pulmonary trunk, with a clonal relationship to right or left head muscles respectively. We thus establish a lineage tree and demonstrate distinct clonal relationships linking groups of head muscles to different parts of the heart, reflecting the posterior movement of the arterial pole during pharyngeal morphogenesis.

123. Cardiac malformations in Pdgfrα knockout embryos are mediated by altered second heart field development

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Platelet-derived growth factors (PDGFs) are known for their role in cardiovascular development. The PDGFR-α expression pattern in normal development was studied with immunohistochemistry and in embryos in which an allele of the PDGFR-α expresses a nuclear localized green fluorescent protein from the PDGFR-α locus. These data showed expression of PDGFR-α in various parts of the heart and in second heart field (SHF) derived mesoderm at the venous pole. We analyzed hearts of Pdgfrα knockout mouse embryos between embryonic day (E) 9.5 and E14.5 for malformations at the venous pole. Immunohistochemical staining, morphometry analysis and 3D-reconstruction were performed to characterize cardiac malformations. The results showed that Pdgfrα-/- have cardiac malformations related to altered addition of myocardium from the SHF. First, Pdgfrα mutants showed hypoplasia and altered differentiation of the sinoatrial node and venous valves. Hypoplasia of the primary atrial septum, mesenchymal cap and dorsal mesenchymal protrusion resulted in failed fusion of the atrioventricular (AV) septal complex. The pulmonary vein (PV) remained connected to the non-incorporated sinus venosus, indicative of development of total abnormal pulmonary venous connection (TAPVC). Second, impairment in the development of the proepicardial organ, epicardial adhesion with severe blebbing and migration of epicardial cells, coincided with hypoplasia of ventricular compact and septal myocardium and AV valve abnormalities. Our results demonstrate that PDGFR-α expression is necessary for addition of the myocardial and mesenchymal components of the SHF at the venous pole of the heart. The data support a crucial role for PDGFR-α in cardiac development, through mesenchymal-myocardial and epicardial-myocardial interaction.

124. Determining the Genetic Requirement of Mouse Castor during Cardiac Development

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To identify the mechanisms that regulate the onset of cardiomyocyte differentiation, we are characterizing the role of the zinc finger transcription factor, Castor. Castor was initially identified for its role in maintaining stem cell competence in the Drosophila dorsal midline, and recently has been shown by studies in our lab to be involved in cardiogenesis. We have demonstrated that Xenopus Castor is expressed in the developing heart and is required for the differentiation of cardiomyocyte progenitor cells at the ventral midline. We have gone on to identify Castor orthologues in mouse and human. In the mouse, we have demonstrated that Castor is expressed during heart development, beginning with a high level of expression in the forming tubular heart and later in the ventricles and atria. To define the specific role of mammalian Castor in cardiac progenitor cells, we have generated a conditional knock-out in ES cells and are now using these to generate mouse strains. In parallel, to determine the fate of Castor positive cells in the developing mouse heart, we have knocked CreERT2 into the Castor locus. Specifically, we will use these novel tools and strains to identify the cells types and structures Castor positive cells give rise to and if they mark a novel cardiogenic precursor population.

125. Mechanism of CASTOR function in early heart development

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An understanding of the molecular pathways that trigger differentiation during heart development is critical for uncovering the pathologies and treatment of congenital heart disease. To address these issues, we cloned and characterized the Xenopus orthologue of a zinc finger transcription factor, CST. In the absence of CST, cells at the ventral midline fail to differentiate and remain as cardiac precursors. Conversely cardiac progenitor cells in the more dorsal portion of the developing heart over-proliferate yet undergo proper cardiac differentiation. Collectively, these defects abrogate the proper formation of the linear heart tube, lead to aberrant heart looping and morphogenesis, and ultimately result in embryonic lethality. To determine the function of CST in the heart at the molecular level, a yeast two-hybrid screen was performed to identify protein partners of Xenopus CST using a cardiac specific library. From this screen we identified congenital heart disease 5 protein (CHD5)/tryptophan rich basic protein (WRB), a protein of unknown function encoded from the genomic locus linked to congenital heart disease in Down Syndrome patients. We have shown that CHD5 is co-localized with CST in Xenopus cardiomyocytes within the developing heart. Critically CHD5 depletion in Xenopus leads to a bifurcation of the heart fields, a phenotype similar to CST depletion. The similarities in phenotype between these co-localized proteins indicate that they may interact directly in regulating cardiac differentiation.

126. Endothelin type-A receptor expression defines a distinct subdomain within the crescent-forming heart field contributing to chamber myocardium

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The endothelin-1 (Edn-1)/endothelin type-A receptor (EdnrA) signaling is now implicated in craniofacial and cardiovascular development. To identify target cells of the ET-1/EdnrA signaling in heart development, we knocked-in lacZ (EGFP) marker genes into the EdnrA locus. In developing heart, lacZ (EGFP) was expressed in cardiac neural crest-derived structures in the outflow tract and pharyngeal arteries. In addition, lacZ (EGFP) expression was also found in the inflow tract at primitive heart tube through looping/ballooning stages. This expression overlapped partially with expression of first heart field marker, Nkx2.5 and Mlc2a, in the ventral side from early heat developing stage. These results suggest that Ednra-lacZ (EGFP)-expressing cells might be a subset of the first heart field. Ednra-lacZ (EGFP)-expressing cells are located in the ventrocaudal region of the crescent/linear heart tube, and then distribute anteriorly along the outer curvature, contributing to the formation of chamber myocardium mainly in the left ventricle and both atria. This apparent upward movement was confirmed by fluorescent dye labeling experiments. These findings indicate how subpopulations within the crescent-forming heart field contribute to coordinate heart morphogenesis through spatiotemporally defined cell movements. Furthermore, Ednra-null embryonic hearts often demonstrate ventricular hypoplasia, indicating that the Ednra-mediated signal is involved in normal myocardial growth and chamber morphogenesis. Ednra-null embryonic hearts also showed decreased bromodeoxyuridine uptake and Erk1/2 phosphorylation at E9.5, indicating that mitotic activity of early cardiomyocytes are downregulated by Ednra-null mutation. These findings lead us to speculate that Edn1/EdnrA may be involved in early myocardial development as a mitogenic signal.

127. The Secondary Heart Field: A Proposed Common Theme Among Vertebrates

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In chick and mouse embryogenesis, a population of cells described as the secondary heart field (SHF) adds both myocardium and smooth muscle to the developing cardiac outflow tract (OFT). Following this addition, at approximately HH stage 10 in chick embryos, for example, the SHF can be identified architecturally by an overlapping seam at the arterial pole, where beating myocardium forms a junction with the smooth muscle of the arterial system. Previously, using either immunohistochemistry or nitric oxide indicators such as diaminofluorescein 2-diacetate, we have shown that a similar overlapping architecture also exists in the arterial pole of zebrafish and some shark species. However, although recent work suggests that development of the zebrafish OFT may also proceed by addition of a SHF-like population of cells, the presence of a true SHF in zebrafish and in many other developmental biological models remains an open question. We performed a comprehensive morphological study of the OFT of a wide range of vertebrates. Our data suggest that all vertebrates possess three fundamental OFT components. A proximal myocardial component, a distal smooth muscle component, and a middle component that in most cases contains overlapping myocardium and smooth muscle surrounding and supporting the outflow valve(s). Because the middle OFT component of avians and mammals is derived from the SHF, these observations suggest that a SHF may be a common theme in vertebrate embryogenesis.

128. The Hox fate map reveals common origin of both the venous and arterial poles of the forming heart tube

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One important issue concerning heart development is the identification of the mechanisms that instruct cardiac progenitors, which contribute to different functional heart compartments. The retinoic acid (RA) signaling pathway is known to be involved in such process. Our recent study showed that RA-deficient embryos (Raldh2-/-) exhibit an expansion of the second heart field (SHF), resulting in arterial and venous poles morphogenesis defects. Some Hox genes have been identified as RA-responsive genes. To assess the role of Hox genes in defining subpopulations of cardiac progenitors we used double in situ hybridization and genetic lineage tracing analysis. We found that Hoxa1, Hoxa3 and Hoxb1 were expressed in the most posterior region of the SHF (pSHF) as early as E7.5, and their expression is dependent on RA signaling. Cre-lox lineage tracing analysis with Hoxa1Cre, Hoxa3Cre and Hoxb1IRESCre alleles reveals that Hoxa1- and Hoxb1-positive cells contribute to both the venous and arterial poles of the forming heart tube. We observed a difference in the contribution of these populations along the proximal and distal outflow tract (OFT) myocardium. Reduction and excess of RA signaling established that this contribution is RA sensitive. Taken together, our results show that Hox genes are likely to be downstream targets of RA in heart development and that cells initially located in the pSHF contribute to the OFT. We are employing transgenic and null mutant alleles to examine effect of increase and loss of Hox activity respectively on heart development.

129. Right ventricle and outflow tract development are dependent on Bmp Type I receptor Alk2 (Acvr1) expression by second heart field cells

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Important roles for Bmp signaling have been implicated in many aspects of cardiac morphogenesis. Expression of Bmp4 by cells of the second heart field (SHF aka anterior heart field, AHF) has been shown to be required for normal outflow tract and great vessel development. It remains unknown to what extent SHF cells themselves respond to such signals. To determine the mechanisms underlying Bmp function in the cells of the second heart field themselves, we have functionally removed their functional Alk2 (Bmp Type I receptor Acvrl) activity using the Mef2c-AHF-Cre driver. Aortico-pulmonary and/or outflow tract septation was abnormal in these conditional Alk2-null embryos, which displayed double outlet right ventricle (DORV) or common arterial trunk (CAT) phenotypes with variable abnormalities in downstream great arteries. All had ventricular septal defects. The single outflow tract valve in CAT mutants had six leaflets. Some mutant embryos of each phenotype survived to birth but became cyanotic and did not live for more than 24 hours. Prior to septation, the mutant embryos could be distinguished by their smaller, abnormally-shaped, right ventricle. Their outflow tract cushion morphology was abnormal but the cushions were not generally hypocellular. Highly regional differences in gene expression were detected in outflow tract myocardium of mutant versus control embryos. These results demonstrate a role in the morphogenesis of the right ventricle and outflow tract for Alk2-mediated BMP signalling in the SHF.

130. Transforming Grwoth Factor beta2 is expressed in the second heart field and is required for outflow tract and right ventricular development

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The cells of the second heart field (SHF) predominantly contribute to the outflow tract (OFT) and the right ventricle (RV). TGFbeta2-deficient embryos had a spectrum of conotruncal defects and defects in valve formation. In situ hybridization at E9.5-E10.5 showed significant gene expression of TGFbeta2 and TGFbeta1 in the splanchnic mesoderm and dorsal mesocardium. Although TGFbeta3 gene was not expressed in the SHF during early heart development, there was a significant overlap of the TGFbeta2 and TGFbeta3 gene expression at E11.5 and onward during heart development. Here, we demonstrated a novel function of TGFbeta2 in SHF. Histological and morphometric analysis at E10.5 indicated altered cardiac looping and significant reduction of the OFT lengthening in TGFbeta2-deficient embryos. Interestingly, simultaneous gene-targeted deletion of both TGFbeta2 and TGFbeta3 exacerbated OFT septation defects and resulted in a smaller RV at E14.5. Since RV was significantly smaller and hypoplastic in both TGFbeta2-null/TGFbeta3-null and TGFbeta2-null/TGFbeta3-haploinsufficient embryos but not in TGFbeta2-haploinsufficient/TGFbeta3-null embryos, these data indicated a dominant role of TGFbeta2 in the development of RV. Consistent with the overlapping gene expression of TGFbeta2 and TGFbeta1 in the SHF the TGFbeta2/TGFbeta1-defcient embryos exhibited cardiac abnormalities and died at E10.5. The cardiac phenotypic characterization of TGFbeta2/TGFbeta1-deficient embryos and how TGFbeta2 regulates SHF function are currently under investigation. Overall, these studies revealed novel function of TGFbeta2 in the SHF and SHF-derived cardiac segments, including OFT and RV. This will provide new understanding of the etiology of conotruncal heart defects and hypoplastic right heart syndrome. (Funding: R01HL92508-Conway-Doetschman; ABRC:0901 & Schneider/Gieszl Award-Azhar_

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

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The theory that the heart ¬arises from two distinct heart fields has suggested important clues as to how the heart is patterned. Determining the contributions of the first heart field (FHF) and second heart field (SHF) cell populations to the fully formed heart is central to understanding heart development and the etiology of congenital heart defects (CHDs). Haploinsufficiency of the transcription factor TBX5 causes Holt-Oram Syndrome, which includes CHDs. Tbx5 expression outlines a region of the heart that is presumed to correspond to the FHF, and Tbx5 is required in the posterior half of the heart. However, it is not known what structures within the heart the Tbx5 lineage gives rise to, or whether it is important to Tbx5's prevalence in CHDs. Using an inducible Cre recombinase inserted at the murine Tbx5 locus, we have mapped the descendents of the Tbx5-expressing FHF lineage and are determining their intersection with the second heart field (SHF) to discern each field's respective contribution to the heart. We find that from the earliest stages of Tbx5 expression, prior to formation of the linear heart tube, the Tbx5 lineage contributes to the posterior segments of the heart, ending at a sharp boundary at the interventricular septum. We propose that in the developing heart these earliest Tbx5 expressing cells define the FHF lineage, and that a strict lineage boundary is created between the FHF and SHF prior to any morphogenic distinction between their descendents. Understanding the contribution of Tbx5 to the FHF, in conjunction with identifying the cardiac gene program regulated by Tbx5, will contribute to our knowledge of heart development and CHDs.

132. Tyrosine Hydroxylase is expressed during early heart development and is required for cardiac chamber formation

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Aims: Tyrosine Hydroxylase (TH) is the first and rate-limiting enzyme in catecholamine biosynthesis. Whereas the neuroendocrine roles of cathecolamines postnatally are well known, the presence and function of TH in organogenesis is unclear. The aim of this study is to define the expression of TH during cardiac development and to gain insight into the role it may play in heart formation. Methods and Results: We studied TH expression in chick embryo by whole mount in situ hybridization and by quantitative RT-PCR, and analyzed TH activity by high-performance liquid chromatography. We used gain and loss-of-function models to characterize the role of TH in early cardiogenesis. We found that TH expression was enriched in the cardiac field of gastrulating chick embryos. By stage 8, TH mRNA was restricted to the splanchnic mesoderm of both endocardial tubes and was subsequently expressed predominantly in the myocardial layer of the atrial segment. L-DOPA and Dopamine induced ectopic expression of cardiac transcription factors and the atrial myosin heavy chain (AMHC1), as well as sarcomere formation. Overexpression of TH led to increased AMHC1 and Tbx5 expression in the ventricular region, and induced bradiarrythmia. Conversely, blockage of Dopamine biosynthesis and loss of TH activity decreased AMHC1 and Tbx5 expression, while exposure to retinoic acid induced TH expression in parallel to that of AMHC1 and Tbx5. Conclusions: TH is expressed in a dynamic pattern during the primitive heart tube formation. TH induces cardiac differentiation in vivo and it is a key regulator of the heart patterning, conferring atriogenic identity.

133. MEF2C is Required in the Anterior Heart Field for Outflow: Tract Endocardial Cushion Remodeling

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The mammalian heart is formed from at least two distinct populations of mesodermal precursors. Cells from the anterior heart field (AHF) are progressively added to the heart at the time of cardiac looping and lineage tracing has revealed that these cells give rise to the right ventricle, outflow tract, and ventricular septum. The MADS box transcription factor MEF2C is required for proper heart development, and targeted inactivation of *Mef2c* in the mouse results in embryonic lethality at midgestation. Mutant embryos display profound abnormalities of the outflow tract and right ventricle. These studies suggest that MEF2C plays an important role in the AHF and its derivatives. Here, we describe the conditional inactivation of *Mef2c* in the AHF. *Mef2c* AHF conditional knockout mice are born at Mendelian ratios, but half die within the first day of life with profound cyanosis. Histological analyses revealed a spectrum of conotruncal defects and the common features of semilunar valve leaflet thickening and membranous ventricular septal defects suggested aberrant remodeling of the outflow tract endocardial cushions. The transcription factor NFATc1 is known to be required for semilunar valve leaflet remodeling and membranous ventricular septal formation. Immunofluorescence studies revealed a marked reduction in expression of NFATc1 in the pro-valve endocardium of the outflow tract in *Mef2c* AHF conditional knockout mice. Interestingly, a previously described intronic enhancer from the *Nfatc1* gene contains a conserved MEF2 site, and mutation of the *Nfatc1* MEF2 site abolished activity of the enhancer in transgenic embryos in vivo. These studies demonstrate that MEF2C is required in the AHF for proper conotruncal development. They further establish *Nfatc1* as a direct transcriptional target of MEF2C in a transcriptional hierarchy controlling semilunar valve leaflet and membranous ventricular septal remodeling.

Session B - Miscellaneous

134. Temperature-controlled Operating Work Station for the Chick Embryo

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During various experimental manipulations in preparation for studies in cardiovascular development chick embryos as a model system are often exposed to an unfriendly environment consisting of a lower humidity and temperature. This is especially true for and thus very critical in studies that involve in vivo imaging of the beating embryonic heart. Although different measures are implemented to prevent a drop in temperature, significant changes of physiological parameters, e.g. heart rate, still remain a challenge. So far, in use are mainly warmed sand boxes, warm air streams or heating blankets or pads to provide some temperature control. Nevertheless in most cases a significant depression of heart rate is inevitable. Here we present a modified PID-controlled heating device that delivers a stable temperature allowing manipulations of the chick embryo maintaining a physiological heart rate over longer time periods. As humidity is not controlled, this system is not a replacement for an environmental chamber for long-term studies, but combines the advantages of temperature control and ease of use for manipulations. Once the embryos are prepared using this new temperature-controlled operating work station, they can be easily transferred for further studies over longer time periods in an environmental chamber with stable conditions for temperature, humidity and oxygen control (1) as it is conducted in our lab. The modified PID-controlled heating device is optimized for chick embryos in shell-less culture but could easily be modified and used also for studies with zebrafish or mouse embryos. (1) Orhan et al. (2007): Microsc Microanal. 13(3): 204-10.

135. Phylogeny of the reptile heart

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The reptile heart constitutes 0.25 % of the body mass and beats at frequencies approximately 5 fold lower than in similar sized mammals and birds. Systemic and pulmonary venous blood is structurally separated by the two atria but mix within the incompletely divided ventricles of snakes, lizards and turtles. Only in crocodiles is there a complete interventricular septum. The ventricle exhibits the most interspecies variation with septa of variable sizes and a slender outline in snakes while broad in turtles. Nevertheless, there is constant volume relationship between the left ventricle (40 %) and the right (60 %) in snakes, lizards and turtles. Accordingly, the inevitably mix, shunt, of blood within the ventricles of these groups seem to be by default predominantly right-to-left, i.e. pulmonary bypass. We therefore argue that the hearts of snakes, lizards and turtles are essentially the same. Python snakes and varanid lizards, however, have mammal-like blood pressures and their ventricular chamber volumes deviate from the 40-60 % relationship and their septa are clearly specialized. The heart of the Tuatara, possibly the evolutionary oldest reptile alive, has ventricular septa that show very little specialization. Crocodiles on the other hand, grouped in the archeosaur clade together with dinosaurs and birds, has the heart that shows the strongest resemblance to the four-chamber heart of mammals and birds. The ventricles of snakes, lizards and turtles then represent the transitional state where pythons and varanid lizards are the advanced exceptions to the rule.

Session B - Transcription Regulation

136. Conditional Disruption of Phosphoregulation of Hand1 Results in Outflow Tract Defects

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For abstract, see in oral presentation section page 27

137. Tbx2 and Tbx3 induce cushion formation and are required for AVC patterning

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138. The Cardiac Transcription Network Driven by the Interplay of Transcription Factors, Histone Modifications and microRNAs

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139. A MEF2C-Myocardin complex directly regulates the cardiac metabolic master regulatory gene Ampka2 through a conserved myocardial-specific enhancer

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140. Inhibition of cardiac myocyte apoptosis by Zac1, an essential cardiac transcription factor

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141. Hand2 loss-of-function in Hand1-expressing cells Reveals Distinct Roles In Heart And Coronary Vascular Development

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142. Direct Reprogramming of Cardiac Fibroblasts into Functional Cardiomyocytes by Defined Factors

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143. Sox4 cooperates with Tbx3 in the regulation of T-box-gene targets

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144. Prdm1, encoding the Blimp1 transcriptional repressor, genetically interacts with Tbx1 during distal outflow tract morphogenesis.

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145. A Direct microRNA Target Screen Reveals Alternate Regions of Complementarity that Mediate Repression in cardiac homeostasis

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146. Interplay of TBX5 and MEF2C during early heart development

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TBX5 is a transcription factor which plays important roles in the development of the heart and upper-limbs. Mutations in this gene produce the inherited disorder Holt-Oram syndrome. During heart development TBX5 associates with various factors including NKX2.5, GATA4, TAZ and orchestrates gene transcription. Here we report a novel interaction between TBX5 and MEF2C leading to a synergistic activation of a cardiac myosin heavy chain (MYH6). We also show that they physically associate through their DNA-binding domains to form a complex on the MYH6 promoter. TBX5 mutants; TBX5G80R and TBX5R279X that produce severe cardiac phenotypes impair the synergy. Morpholino-mediated knockdown of Tbx5 and Mef2c in zebrafish suggests that the genetic interaction of these proteins is not only required for MYH6 expression, but also is essential for the early stages of heart development and survival.

147. Identification of Twist1 Target Genes in Heart Valve Development

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Congenital heart defects, including valve malformations, are among the most common birth defects in the United States. During early embryonic heart formation, valvulogenesis begins with the formation of endocardial cushions (ECs) in the atrioventricular canal and outflow tract as a result of an endothelial to mesenchymal transition (EMT) of the endocardium. EC mesenchymal cells are highly proliferative, migratory, and reside in a primitive ECM. The bHLH transcription factor Twist1 is expressed preferentially in the EC mesenchyme and promotes proliferation, migration, and a primitive ECM of EC cells in culture, yet the mechanism by which this occurs remains unclear. We hypothesize that Twist1 maintains EC mesenchymal cell population by directly activating genes promoting cell proliferation, migration, and a primitive ECM. The transcription factor, Tbx20 and adhesion molecule, Cadherin-11 (Cdh11) are two candidate genes modulated by manipulation of Twist1 in EC explant cultures. Regulatory regions containing E-boxes, consensus-binding sites for Twist1, were identified in Tbx20 (Tbx20boxA) and Cdh11 (Cdh11-Intron1). Luciferase assays confirmed that Twist1 drives expression from both regulatory regions, and site-directed mutagenesis of the E-box in Tbx20boxA abolishes Twist1 activation. Additional candidate downstream targets have been identified through Affymetrix microarray analysis of MC3T3-E1 preosteoblast cells treated with control siRNA or Twist1 siRNA. Loss of Twist1 results in decreased expression of genes associated with cell proliferation, migration, and components of the ECM. These experiments will further elucidate transcriptional hierarchies downstream of Twist1 that control proliferation, migration, and primitive ECM gene expression in EC mesenchymal cells.

148. Systems level analysis of cardiac transcription factor networks using DamID

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We have begun to formally dissect mammalian cardiac regulatory pathways at a systems biology level using genomic tools and genetically manipulated mice and cells. Our initial focus is to develop the Dam methylation identification (DamID) method for genome-wide cardiac transcription factor (TF) target gene identification, a technique that overcomes some of the limitations of the ChIP-on-chip and ChIP-seq techniques currently used for similar purposes. We have performed first pass proof-of-principal experiments to validate the DamID method, applying it first to a differentiated atrial cardiac cell line (HL-1) and isolating data sets for Nkx2-5, Gata4, Isl1, Tbx5, Tbx20, Tbx1 and Tbx2. Data sets have been collected largely using Affymetrix promoter tiling microarrays, although for Nkx2-5 targets we have also interrogated whole-genome arrays for chromosomes 4, 11 and 17. We will provide an update on our explorations thus far. The DamID method should provide a valuable template and complement to ChIP data for analysis of gene regulatory networks in mammalian cardiac development and physiology, with applications to understanding cardiac disease mechanism and progression.

149. P38alfa-mediated Cited2 phosphorylation is required for Pitx2 expression in cardiomyocytes

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Cited2 encodes for a transcriptional co-factor that appears to participate in many biological processes through negative or positive regulation of p300/CBP-dependent gene transcription. Cited2 binds Tfap2 and CBP/p300, a coactivator phosphorylated by the MAPK P38.Very recent reports show that Cited2 and Tfap2 were detected at the Pitx2c promoter in embryonic hearts and propose that Cited2 acts directly upstream of Pitx2c. In addition, embryos null for either Cited2 or Pitx2c develop similar cardiac defects with abnormal left-right has been established to causeαpatterning. Finally, loss of function of p38 embryonic lethality at midgestation due to a placental defect, similarly than Cited2 -/- null mouse embryos. To further analyse Cited2-mediated Pitx2 transcriptional regulation we used mouse immortalised p38alfa-/- and wild-type embryonic cardiomyocytes cell lines obtained from E 9.5. Our results demonstrated that p38alfa deficiency abolish expression of the three Pitx2 isoforms, Pitx2a, b, and C. In addition, lack of p38alfa impairs Cited nuclear localization in cardiomyocytes, colocalising with vimentin at the perinuclear region, while P300 nuclear localisation was not altered. Taken together, we mediated Cited2 phosphorylation is required to regulate–propose that p38alfa- its nuclear localisation and allows Pitx2 expression in embryonic cardiomyocytes.

150. Wt1 and retinoic acid signaling in the subcoelomic mesenchyme control the development of the pleuropericardial membranes and the sinus horns

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The cardiac venous pole is a common focus of congenital malformations and atrial arrhythmias, yet little is known about the cellular and molecular mechanisms that regulate its development. The systemic venous return myocardium (fetal sinus horns) forms only late in cardiogenesis from a pool of pericardial mesenchymal precursor cells. Here, we show that expression of the Wilms tumor 1 gene (Wt1) in the subcoelomic mesenchyme surrounding the cardinal veins is important for sinus horn development. In Wt1-deficient hearts, the cardinal veins fail to expand into the pericardial cavity and to differentiate to myocardium, but become embedded laterally in the pleuropericardial membranes. These membranes remain tethered to the lateral body wall by the persisting subcoelomic mesenchyme, a finding that correlates with decreased apoptosis in this region. Expression of the aldehyde dehydrogenase family 1, subfamily A2 gene (Raldh2) was lost from this mesenchyme in Wt1-deficient embryos, and Raldh2- and Wt1-mutant mice display similar defects of the venous pole and pericardium, suggesting that retinoic acid signaling mediates the function of Wt1 in this tissue. We show by lineage tracing studies that the Wt1-positive subcoelomic mesenchyme does not present a cellular source of sinus venosus progenitors, indicating that formation of pericardium and sinus horns depends on the expansion and correct temporal release of the pleuropericardial membranes by apoptosis from the underlying subcoelomic mesenchyme. These results provide novel insight into the genetic and cellular pathways regulating the posterior extension of the mammalian heart and the formation of its coelomic lining.

151. Cardiac Gene regulatory network driving differentiation in the drosophila embryo

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A great number of reports have demonstrated a clear conservation of genetic control of cardiogenesis, from Drosophila to mammals. However, what the involved transcription factors control in terms of downstream gene networks to control the successive steps that progressively drive the diversification and the differentiation of cardiomyocytes, largely remains unknown. Still, a detailed understanding of cardiac differentiation processes should provide important insights about normal and pathological heart development. Our research projects use genetics, developmental biology and genomics to decipher cardiac Gene Regulatory Networks (GRN) involved in cardiac cell diversification and differentiation in Drosophila. Our approach also includes the development and use of bio informatics tools to unravel the cis regulatory code from set of co expressed genes. In addition, we are interested in understanding how shape and function are acquired downstream the cardiac GRN. Reverse genetic screens are under way to identify genes involved in establishment of cardiac tube shape and acquisition of automatic beating.

152. MiR423-5p as a circulating biomarker for heart failure

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Rationale: Aberrant expression profiles of circulating microRNAs (miRNAs) have been described in various diseases and provide high sensitivity and specificity. We explored circulating miRNAs as potential biomarkers in patients with heart failure (HF). Objective: The goal of this study was to determine whether miRNAs allow to distinguish clinical HF not only from healthy controls but also from non-HF forms of dyspnea. Methods and results: A miRNA array was performed on plasma of 12 healthy controls and 12 HF patients. From this array we selected 16 miRNAs for a second clinical study in 39 healthy controls and in 50 cases with reports of dyspnea, of whom 30 were diagnosed with HF and 20 were diagnosed to have dyspnea due to non-HF related causes. This revealed that miR423-5p was specifically enriched in blood of HF cases and receiver-operator-characteristics (ROC) curve analysis showed miR423-5p to be a diagnostic predictor of HF, with an area under the curve of 0.91 ($p<0.001$). Five other miRNAs were elevated in HF cases, but also slightly increased in non-HF dyspnea cases. Conclusion: We identify 6 miRNAs that are elevated in patients with HF, among which miR423-5p is most strongly related to the clinical diagnosis of HF. These 6 circulating miRNAs provide attractive candidates as putative biomarkers for HF.

153. Bioinformatics approach to identify direct downstream T-box target genes

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T-box genes have been involved in early cardiac lineage determination, chamber specification, valvuloseptal development and diversification of the specialized conduction system in vertebrate embryos. This gene family is present in all metazoans, and they are expressed throughout embryonic development in a spatial-temporal coordinated manner. They share a T-box DNA binding domain and many can bind a T-site consensus sequence on DNA. Mutations in T-box genes are responsible for developmental defects with a dosage dependent severity. Particularly, TBX1, has been identified as a critical gene in the chromosome 22q11 region deleted in individuals with velo-cardio-facial/DiGeorge syndrome characterized by craniofacial and heart defects. To find direct transcriptional target genes for Tbx1, we performed SELEX (Systematic Evolution of Ligands by Exponential enrichment) with the T-box portion of the murine Tbx1 protein. A dimer and also monomer site of AGGTGTGA was identified. The site is highly similar to the published Brachyury (T protein) binding site. With this in mind we did a bioinformatics analysis searching for this site in regions of the mouse genome with high evolutionary conservation. We identified 200 sites highly conserved in vertebrates and 500 belonging to the next level of conservation. Many could be direct downstream targets of several T-box genes. Ones expressed in heart were Bhlhe40, ESSRG, Hiflan, Kcnd2, BMP10, Runx11, Slc8a1, Vcl and Egr3, among others. This coupled with ChIP-seq or other ChIP type experiments could help narrow possible candidates to pursue.

154. Regulation of conduction genes by TBX3

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Previous studies showed that T-box transcription factor TBX3 is important for the formation of the cardiac conduction system. Microarray analysis in transgenic mice with TBX3 over-expression in the atria showed that Scn5a and Scn10a, two important cardiac sodium channel genes, are down-regulated. Down-regulation of Scn5a may predispose to channelopathies, and recently Scn10a has been linked to cardiac conduction in genome-wide association studies. Hence, the regulation of these genes is important for normal cardiac conduction. In order to find out more about the function of TBX3 in gene regulation, we performed an in vivo TBX3 ChIP-seq (chromatin immunoprecipitation coupled to massively parallel sequencing) in adult mouse hearts with TBX3 over-expression. Using this ChIP approach we have now been able to map all potential and open TBX3 binding sites, in vivo, within and far beyond the Scn5a and Scn10a encoding region. In addition to genome-wide mapping of protein binding, ChIP-seq also allows us to predict enhancers in the genome. By means of the occupancy of TBX3 in Scn5a and Scn10a genes, a number of sequences in these genes were chosen and analysed in vitro using a luciferase reporter assay. Taking this approach we identified several potential enhancers that could be down-regulated by TBX3 and up-regulated by addition of GATA4 and NKX2.5. We go on and verify these binding sites as being occupied by TBX3 and GATA4/NKX2.5/(p300) during embryonic development. We are currently examining the spatiotemporal expression patterns these enhancers control during embryogenesis.

155. Gli2 and Mef2C interact on genetic and protein levels during stem cell differentiation

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Gli2 is a primary mediator of the sonic hedgehog (Shh) signaling pathway during myogenesis and neurogenesis in developing embryos. Previously, Gli2 has been shown to play a role in cardiomyogenesis and skeletal myogenesis in vivo and to induce cardiac and skeletal myogenesis in vitro in P19 cells, an established stem cell model for muscle and neuronal cell differentiation. We found that in addition to myogenesis, expression of Gli2 resulted in the induction of neurogenesis in P19 cells. This is the first observation of the neurogenic effect of Gli2 in stem cells. Similar to Gli2, Mef2C is also important in myogenesis and neurogenesis in vivo, and its expression resulted in induction of cardiac and skeletal muscle as well as neurons in P19 cells. We found that Gli2 and Mef2C upregulated each other's transcription by directly binding to their respective gene elements, indicating a novel positive regulatory loop between Gli2 and Mef2C. Moreover, we found that Gli2 and Mef2C form a protein complex during P19 cellular differentiation. Gli2 and Mef2C proteins showed synergistic activation of the Nkx2.5 promoter, a factor playing a major role in cardiomyogenesis both in vitro and in vivo. Our findings shed light on transcriptional regulation as well as protein complex formation and function during myogenesis and neurogenesis by Shh signaling in vitro.

156. The Iroquois homeobox gene 3 regulates functional maturation of the His-Purkinje Network

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Integral to cardiac function are the fast-conducting myocytes of the His-Purkinje network that drive ventricular activation. Here we show that the Iroquois homeobox gene 3 (Irx3) is essential for establishing conduction properties of this specialized cellular network. Genetic ablation of Irx3 in mice resulted in impaired ventricular activation, measured as QRS prolongation and bundle branch block. The zebrafish homolog Ziro3a is highly expressed in the ventricle and atrioventricular canal. Optical mapping of its morpholino knockdown also showed slowed and asynchronous impulse propagation. Further studies in Irx3 null mice revealed that impaired conduction is attributable to reduced cellular excitability and altered intercellular coupling. In the absence of Irx3, we observed changes in ion channel genes that underlie the depolarizing phase of the action potential, and ectopic connexin 43 (Cx43) expression in the proximal bundle branches. Adenoviral overexpression of Irx3 in neonatal ventricular myocytes led to marked decrease of Cx43 mRNA and protein. We have determined that Cx43 is a direct transcriptional target, and that Irx3 represses Cx43 activation by Nkx2-5 and Tbx5. Together these results suggest that Irx3 is required for His-Purkinje functional maturation as an evolutionarily conserved regulator of impulse conduction. Using a 3myc-6his-Irx3 knockin mouse line, we are currently investigating additional Irx3 protein partners and its endogenous consensus DNA binding sequence.

157. Transcriptional Regulation of Fgf10 Gene in the Anterior Heart Field

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The anterior part of the second heart field (AHF) contributes to the arterial pole of the heart. In the AHF, Fgf10 is expressed and functions for formation of the outflow tract and right ventricle. To elucidate signaling cascade in the AHF, we identified and analyzed AHF enhancer of Fgf10 gene. Fgf10 AHF-enhancer has multiple binding sites for Nkx2.5, which is a homeobox transcription factor and is predominantly expressed in the heart. EMSA showed Nkx2.5 binding to those sites, and Fgf10 AHF-enhancer activity was ectopically induced in the single ventricle of Nkx2.5 mutants, suggesting that Nkx2.5 is a suppressor for Fgf10 AHF-enhancer. We next tested Isl1 regulation to the enhancer. Isl1 is a LIM-homeobox transcription factor, and a key regulator for the AHF development. Isl1 shares some of its binding sites with Nkx2.5. In Isl1-overexpressing heart, Fgf10 AHF-enhancer activity was ectopically induced in the heart. Taken together, we conclude that Nkx2.5 and Isl1 bind to same sites in Fgf10-AHF enhancer, and they probably regulate Fgf10 transcription competitively in the heart.

158. Platelets play an essential role in separating the blood and lymphatic vasculatures during embryonic angiogenesis

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Rationale: Several mutations that impair the development of blood lineages in the mouse also impair the formation of the lymphatic vasculature and its separation from the blood vasculature. However, the basis for these defects has remained unknown because the mutations characterized affect more than one blood lineage. Objective: We tested the hypothesis that megakaryocytes/platelets are required for the formation of the lymphatic vasculature and its separation from the blood vascular system. Methods and Results: We characterized the vascular patterning defects of mice deficient for the homeodomain transcription factor Meis1, which completely lack megakaryocyte/platelets. Meis1 null embryos fail to separate the blood and lymphatic vasculature, showing blood-filled primary lymphatic sacs and superficial lymphatic vessels. To test the involvement of megakaryocytes/platelets in this phenotype, we generated megakaryocyte/platelet-specific deficient mice by targeted lineage ablation, without affecting other blood lineages. This model reproduces the lymphatic/blood vasculature separation defects observed in Meis1 mutants. A similar phenotype was induced by antibody-mediated ablation of circulating platelets in wild type mice. Strong association of platelets with vascular endothelium at regions of contact between lymphatic sacs and veins confirmed a direct role of platelets in the separation of the two vasculatures. Conclusions: In addition to their known protective function in the response accidental vascular injury, platelets are also required during embryonic lymphangiogenesis for the separation of the nascent lymphatic vasculature from blood vessels.

159. Co-operative and synergistic actions of Tbx5 and MRTF-B in zebrafish cardiogenesis

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In response to hemodynamic forces, vascular endothelial cells remodel their cytoskeleton and change their gene expression profiles. Alterations of cardiac hemodynamics produce defects in heart morphology and gene expression. Hence, formation of a functional heart is dependent on a tight interplay between genetics and hemodynamics, both of which are emerging as indivisible parameters for cardiogenesis and circulatory homeostasis. Nonetheless, a molecular link between them is unknown. Tbx5, a key transcription factor, also functions as an epigenetic factor, making a robust transcriptional activation complex with MRTF-B in response to the heartbeat. Our data argue for the importance of a novel epigenetic mechano-transduction pathway mediated by heartbeat-dependent nucleo-cytoplasmic shuttling. We found that correct cyto-architecture and cardiomyocyte contraction were also dependent on the heartbeat, revealing physical force as an epigenetic morphogen.

160. Characterization of cardiac enhancers of the Drosophila Dorsocross T-box gene cluster

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A critical aspect of heart formation is the precise control of the spatio-temporal expression of key regulatory genes of the cardiac gene regulatory network. Members of the T-box gene family, which encode transcription factors, are crucial components of this network in both vertebrate and invertebrate organisms. In Drosophila, the T-box genes Dorsocross (Doc1-3) act in conjunction with the Nkx2-5 family-founding member tinman (tin) and the GATA4 homologue pannier (pnr) to specify cardiac progenitors at an early stage of cardiogenesis. Accordingly, these cardiogenic factors are co-expressed in the early cardiogenic mesoderm. However, during subsequent stages tin represses Doc and, consequently, Doc and tin assume mutually exclusive expression patterns in developing cardioblasts. Thus, Doc gets restricted to the myocardial cells that will form the inflow valves and tin to the working myocardial cells. For a more detailed view of the molecular pathways of cardiac specification and heart cell diversification we have scanned the entire Doc locus of the Doc1-3 gene cluster for enhancers using a transgenic reporter gene approach. We have identified regulatory elements for the expression in the early cardiogenic mesoderm and others that drive expression of Doc in inflow valve-forming cardioblasts. ChIP-on-Chip data suggest that these elements are bound by Tin in vivo. We are currently investigating whether this binding is functional in an activating and repressing fashion, respectively, and which other transcription factors and signaling effectors may target these elements.

161. Genome-wide identification of NKX2-5 binding sites and target genes in the developing heart using ChIP-chip

Catherine Shang*, Stuart Smith, Norma Towers, Tim Mohun

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162. Etv2 regulation of endothelial development in the Xenopus embryo.

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Transcription factors of the ETS family are important regulators of endothelial gene transcription. We have determined that one of the ETS family members, etv2, is essential for vascular development in the Xenopus embryo. Loss of etv2 results in loss of detectable expression of every vascular marker examined, including all endothelial transcription factors. In overexpression experiments, etv2 is sufficient to activate transcription of a large range of endothelial markers, again including endothelial transcription factors. These results suggest that etv2 functions at the very top of the transcriptional hierarchy that controls endothelial cell specification. We want to further explore the mechanism by which etv2 regulates endothelial gene expression during vascular development. Overexpression of etv2 specifically activates endothelial genes, even in non-mesodermal tissues like gut endoderm or ectodermal explants. The DNA binding site specificity of etv2 alone is not sufficient to explain how the protein can be so precise in activating endothelial genes. We hypothesize that etv2 cooperates with other transcription factors and these co-regulatory partner proteins provide at least some additional specificity. First, we are using a candidate approach in an effort to identify functional partner proteins. Second, we are using structure-function studies to dissect a unique conserved sequence in etv2 that may allow for protein interactions. Finally, we will perform co-immunoprecipitation followed by Mass Spec analysis, to identify proteins that physically complex with etv2 in the cell. Since no functional partners of etv2 have been identified at this time, our proposed studies will broaden our understanding of the mechanisms of transcriptional regulation by ETS transcription factors during endothelial development.

163. The ETS family protein, etv2, is necessary and sufficient for activation of the endothelial transcription factors, fli1, elk3 and sox18.

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Transcription factors of the ETS family play an extremely important role in regulation of endothelial and hematopoietic genes. We have identified and characterized the Xenopus orthologue of the ETS transcription factor, etv2/er71. Expression analysis shows that etv2 transcripts are highly expressed in hematopoietic and endothelial precursor cells in the Xenopus embryo. In gain-of-function experiments, overexpression of etv2 strongly activates expression of vascular markers in ectopic regions of the Xenopus embryo using multiple assays including microarray analysis, quantitative RT-PCR and whole mount in situ hybridization. Upregulated genes include a number of endothelial and hematopoietic transcription factors. Inhibition of etv2 function using antisense morpholino oligonucleotides results in dramatic reduction in expression of endothelial marker genes, but expression of hematopoietic markers are not affected. To investigate the potential of etv2 to directly activate endothelial genes, we utilized a steroid inducible etv2 fusion protein, in the presence or absence of protein synthesis inhibition, demonstrating that the endothelial transcription factors, fli1, elk3, and sox18 are direct targets of etv2 regulation. These studies place etv2 at the beginning of the transcriptional program essential for development of the endothelial lineage in the Xenopus embryo. However, these studies do not explain how etv2 directly activates endothelial genes or the regulatory regions of target genes where etv2 binds. To approach these questions we have constructed an epitope-tagged etv2 for use in chromatin immunoprecipitation experiments with the ultimate goal of revealing novel etv2-specific cis-regulatory elements on target endothelial genes.

164. Drosophila microRNA let-7 deficiency results in compromised heart function

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MicroRNAs (miRNAs) are a class of highly conserved non-coding RNAs that have been recently recognized as majorly implicated in cardiovascular diseases. We are using the genetic power of the fly and an optical method for analyzing and quantifying heart contraction parameters to investigate the role of miRNAs in the adult heart function. A cardiac specific miRNA expression profile revealed let-7 as one of the miRNAs expressed in the adult heart. Investigating the requirement for let-7 in cardiac physiology, we found that let-7 mutants display a slower, highly arrhythmic heartbeat, including a high incidence of non-beating (asystoly), as well as structural defects reminiscent of 'hypertrophy'. To better characterize the role of let-7 in the heart, we performed heart-specific Q-PCR for many (direct or indirect) candidate targets of let-7, including ion channel genes. Indeed, we observed that several genes that have previously been found to be critical for heart function are up-regulated in let-7 hearts, including the Calcium channel cacophony, the K-ATP channel subunit dSur and the Ks channel KCNQ. These results demonstrate the importance of let-7 miRNA in the regulation and maintenance of adult cardiac function. We are in the process of investigating the functions of other cardiac-expressed miRNAs in the Drosophila heart model.

165. Functional analysis of genomic regions identified by genome-wide Tinman ChIP-chip in Drosophila

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The formation of the heart and other musculatures from the dorsal mesoderm of Drosophila has provided an excellent model for dissecting how signal molecules and transcription factors cooperate to control organogenesis. Genetic studies identified the NK homeodomain transcription factor Tinman (Tin) as a key regulator in this system. In the absence of tin function, there is a complete loss of all dorsal mesodermal derivatives, including cardiac, visceral and certain somatic muscles. However, so far relatively few direct target genes of Tin have been identified. To better understand the functions of tin in the regulatory network, we performed whole-genome ChIP-chip analysis for Tin in vivo binding sites with embryos of two distinct developmental periods, which cover early cardiac induction and the specification of various types of heart progenitors, respectively. Among the ca. 3500 partially overlapping Tin-bound regions from the two stages several dozens of highly prioritized regions were chosen for functional analysis in both wild type and tin mutant embryos. The Tin-binding sites of several fragments were mutated to examine their roles in the respective enhancer activities. Our results show that most tested Tin-bound regions are able to drive reporter expression in the mesoderm or distinct muscle tissues, and often their enhancer activities completely or partially depend on the presence of functional Tin protein. Our poster will focus on Tin-targeted enhancers and some of their associated genes that are active during various stages of cardiogenesis.

166. Transcriptional repression by the highly redundant Irx4 and Irx5 transcription factors maintains the normal growth properties of the heart.

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The growth of the heart around birth is strictly regulated, especially as the various cell types transition from a fetal proliferative phenotype to a mature differentiated program. Here we show that in mice lacking the Iroquois homeobox (Irx) transcription factors Irx4 and Irx5 the growth properties of the heart are disrupted. Overall, neonatal lethality was observed, with severe myocardial disarray especially pronounced in the interventricular septum. Furthermore, the monolayer properties of the endocardium were disrupted, occasionally leading to thickening of the endocardium up to 20 layers, mimicking endocardial fibroelastosis, a human disease of unknown etiology. Gene profiling revealed that Irx4 and Irx5 are highly redundant, and in their combined absence hundreds of genes are derepressed. Statistical overrepresentation of genes implicated in chromatin remodeling and RNA metabolism were observed, indicating that a broad program incorporating transcriptional and post-transcriptional regulation is affected in Irx4;Irx5 double KO hearts. This shows that the combined role of Irx4 and Irx5 is transcriptional repression of gene programs that regulate the growth and functional properties of the heart. Other Irx genes are also highly redundant, and our preliminary results from Irx3;Irx4 double knockout mice indicate lethality by mid-gestation. These findings have implications for the regulation of growth properties in organogenesis, as well as in the pathology of cardiac disease.

167. FOXO3a negatively regulates the transcriptional activity of Dok-5

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A clonal derivative named P19CL6 has been isolated from pluripotent P19 mouse embryonic carcinoma cells, and this subline efficiently differentiates into beating cardiomyocytes when treated with 1% dimethyl sulfoxide (DMSO). It offers a valuabe model to study cardiomyocytes differentiation in vitro. Comparative proteomic analysis was used to characterize the protein profiles associated with the DMSO-induced cardiomyocytes differentiation of P19CL6 cells. We also found that Dok-5 expression level significantly increased during this process. Dok-5 is a member of the insulin receptor substrates family, which play important roles in the cellular growth, signaling, and survival of cells. But the mechanism of Dok5 upregulation is still unclear. In this study, we first mapped the transcription start site of the mouse Dok-5 gene and characterized its promoter regions. Truncation and mutation analysis of the Dok-5 promoter identified the forkhead binding element responsible for the repression of Dok-5 promoter region. The co-localization of FOXO3a and Dok-5 in the mouse heart allows FOXO3a to be a transcriptional regulator of Dok-5. Electrophoretic mobility shift assay and chromatin immunoprecipitation assay confirmed that FOXO3a could bind to the Dok-5 promoter, accompanied by FOXO3a translocation from the nucleus to cytoplasm. FOXO3a overexpression could inhibit Dok-5 promoter activity. Silencing FOXO3a expression by siRNA upregulated the expression of Dok-5. Our results provide the first evidence that FOXO3a, the PI3K/PKB downstream substrate, acts as a transcriptional repressor to inhibit the expression of Dok-5.

168. Pitx2 Insuficiency Leads To Atrial Remodelling Prone To Arrythmogenesis

Ana Chinchilla (1), Daimi Houria(1), Estefania Lozano-Velasco (1), Jorge N Dominguez (1), Ricardo Caballero (2), Eva Delpón (2), Juan Tamargo (2), Leif Hove-Madsen (3), Amelia E Aranega (1), Diego Franco (1)
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Pitx2 is a homeobox transcription factor that plays a pivotal role in the early left/right determination, downstream of the nodal/lefty signaling pathway. Pitx2 expression is confined to the left side of the embryo within the lateral plate mesoderm and it remains to be expressed in the cardiac crescents and early heart tube. Germ line Pitx2 loss-of-function mouse mutants displayed early embryonic lethality with severe cardiac malformations, demonstrating the importance of Pitx2 during cardiogenesis. Recently, genome-wide association studies have independently reported several risk variants on chromosome 4q25 that are strongly associated with atrial fibrillation. These variants did not show association to known risk factors for atrial fibrillation such as obesity, hypertension or myocardial infarction, but are adjacent to the gene PITX2, providing new links with cardiac physiopathology. We demonstrate herein that PITX2C expression is significantly impaired in human patients with sustained atrial fibrillation. We also report morphological, molecular and electrophysiological characterization of atrial and ventricular chamber specific Pitx2 conditional mouse mutants. Electrophysiological studies in atrial, but not ventricular, Pitx2 chamber-specific adult hearts reveal AV block and alteration of the action potential. Furthermore we have evidences that lack of Pitx2 in the developing and adult atria impairs ion channel expression in line with the electrophysiological findings. Such ion channel impairment is, in part, modulated by miR-1 misexpression. Thus, we provide herein compiling evidences of Pitx2 as an upstream transcriptional regulator which mediates distinct signaling pathways resulting in cellular and molecular changes that are prone to lead arrythmogenic events, such as atrial fibrillation. This work is supported by the VI EU Integrated Project “Heart Failure and Cardiac Repair” LSHM-CT-2005-018630 and by the CNIC grant CNIC-08-2009.

SATURDAY 22 MAY - SESSION C

Time: 13.05 - 14.30 hours
Location: Marble Hall
1st floor and basement

Session Topics

3D-Techniques & Imaging	[posters 169 - 183]
Cardiac Growth	[posters 184 - 192]
Conduction System	[posters 193 - 203]
Genetics & Cardiovascular Malformations	[posters 204 - 246]

Session C: 3D-Techniques & Imaging

169. 3D Modelling of heart development: looking to the future
Tim Mohun
Developmental Biology Division, MRC National Institute for Medical Research, London, UK
For abstract, see in oral presentation section page 59

170. Imaging in the Embryonic Heart: morphodynamic studies with Optical Coherence Tomography (OCT)
Mesud Yelbuz
Pediatric Cardiology & Intensive Care, Hannover Medical School, Hannover, Germany
For abstract, see in oral presentation section page 60

171. How to quantify in 3D: imaging of gene expression patterns and morphogenetic parameters in the embryonic heart.
Jan M. Ruijter, Alexandre T. Soufan, Jaco Hagoort, Bouke A. de Boer and Antoon F.M. Moorman
Department of Anatomy, Embryology & Physiology, Heart Failure Research Center , Academic Medical Center, Amsterdam, NL
For abstract, see in oral presentation section page 61

172. Automated fitting of histological sections into a high resolution 3D reference model of a developing mouse heart
Bouke A de Boer* (1,2), Frans PJM Voorbraak (2), Maurice JB van den Hoff (1), Antoon FM Moorman (1), Jan M Ruijter (1)
(1) Heart Failure Research Center, Dept. Anatomy, Embryology & Physiology, Academic Medical Center, Amsterdam (2) Dept. Medical Informatics, Academic Medical Center, Amsterdam, NL
For abstract, see in oral presentation section page 62

173. From 3D-reconstruction to interactive 3D-pdf; a protocol for biologists.
Jaco Hagoort*, Alexandre Soufan, Bouke de Boer, Antoon Moorman
Department of Anatomy, Embryology & Physiology, Heart Failure Research Center, Academic Medical Center, Amsterdam, NL
For abstract, see in oral presentation section page 63

174. Rotationally acquired 4D-optical coherence tomography of embryonic chick hearts using retrospective gating on the common central A-scan

Jan Thommes # (1), Christoph M. Happel *# (2), Lars Thrane (3), T. Mesud Yelbuz (2) # authors contributed equally
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In vivo analysis of cardiac physiology and non-invasive imaging of the beating early embryonic heart in 2 and 3D remain a challenge in cardiovascular development research. 3D-imaging of the beating heart relies on gating of the acquired images according to the cardiac cycle. Mostly ECG triggering is used for this purpose, e.g. in MRT or CT of human hearts. For visualization of embryonic chick hearts with high-resolution optical coherence tomography (OCT), a gating trigger generated by laser Doppler velocimetry has been successfully demonstrated (1). But this takes time and adds to system complexity. More recently, retrospective gating technologies were described (2; 3). In these studies, a time series of 2D images at several positions along the heart volume was used to produce a time sequence of 3D volumes of the beating heart. Rearrangement and validation of the asynchronously acquired input data are based on image similarity of adjacent pictures and a perpendicularly recorded B-scan or a repetitive low-resolution 3D-volume-scan. In our study, we introduce a novel technique that uses a rotational scan mode of a swept-source OCT system (Thorlabs) for imaging the beating embryonic heart. The central A-scan is identical with the rotational axis. Thus, the heart phase can be synchronized in every rotated image plane from the central A-scan. A rotation of 180° allows to create a complete 4D dataset of the beating embryonic chick heart. (1) Jenkins et al. (2007): J Biomed Opt. 12(3):030505 (2) Liu et al. (2009): J Biomed Opt. 14(4):044020. (3) Gargesha et al. (2009): Opt Express. 17(13):10786-99.

175. Integration of an optical coherence tomography (OCT) system into a new environmental chamber to facilitate long term in vivo imaging of cardiovascular development in higher vertebrate embryos

Lars Thrane# (1), Jan Thommes# (2), Christoph M. Happel (3), Jörg Männer (4), T. Mesud Yelbuz* (3) # authors contributed equally
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High-resolution 3-D in vivo imaging of embryonic development over long periods of time under constant physiological conditions (e.g. temperature, humidity) was a challenging task for researchers working on early cardiovascular development. Without appropriate maintenance of temperature, for example, the embryonic heart rate declines rapidly and often results in an increase in regurgitant flow. However, preload, afterload, and the effects of blood flow on cardiac morphodynamics are critical parameters that have to be examined more closely for a better understanding of normal and abnormal cardiovascular development. Here we demonstrate, to the best of our knowledge, the first realization of an optical coherence tomography (OCT) system integrated into a new environmental incubation chamber (EIC) to facilitate real-time in vivo imaging of cardiovascular development in chick embryos. The EIC provides stable conditions for embryonic development with respect to temperature, humidity, and oxygen levels. An OCT probe is integrated into the EIC and facilitates visualization of embryos at micrometer resolution, including the acquisition of M-mode, Doppler OCT, and Doppler M-mode data.

176. Three-dimensional measurement and visualization of morphogenesis applied to cardiac embryology

Alexandre T. Soufan* (1), Gert van den Berg (1), Perry D. Moerland (2), Maarten M. G. Massink (1), Maurice J. B. van den Hoff (1), Antoon F. M. Moorman (1) and Jan M. Ruijter (1)
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Volume growth and proliferation are key processes in heart morphogenesis, yet their regionalization during development of the heart has been described only anecdotally. To study the contribution of cardiomyocyte proliferation to heart development, a quantitative reconstruction method was designed, allowing the local mapping of this morphogenetic process. First, a morphological surface reconstruction is made of the heart, using sections stained specifically for cardiomyocytes. Then, by a comprehensive series of image processing steps, local three-dimensional (3D) information of proliferation is obtained. These local quantitative data are then mapped onto the morphological surface reconstruction, resulting in a reconstruction that not only provides morphological information (qualitative), but also displays local information on proliferation rate (quantitative). The resulting 3D quantitative reconstructions revealed novel observations regarding the morphogenesis of the heart.

177. High-resolution 3-D imaging of the anatomy of normal and malformed embryonic chick hearts through Micro-Computed Tomography (Micro-CT) of critical point-dried heart specimens

Christoph M. Happel* # (1), Christian Klose # (2), Gabriele Witton (2), Gian L. Angrisani (2), Soenke Wienecke (3), Stephanie Groos (4), Friedrich-Wilhelm Bach (2), Dirk Bormann (2), Jörg Männer (5), T. Mesud Yelbuz (1) # authors contributed equally
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In the past, several non-destructive imaging techniques (e.g. Magnetic Resonance Microscopy, Optical Coherence Tomography) were tested for their suitability to visualize the 3-D morphology of embryonic hearts. Most of these imaging tools have their drawbacks with respect to resolution and penetration depth. Here we present, to the best of our knowledge, the first high-resolution 3-D images at 10 µm level of normal and malformed embryonic chick hearts (double outlet right ventricle, juxtaposition of atrial appendages) generated by Micro-CT examination of critical point-dried heart specimens. Day 9 embryonic chick hearts are shown in rotating 3-D images and in fly-through movies. Cardiac anatomy is demonstrated in great details with respect to myocardial fiber arrangement and trabeculations as well as atrioventricular and semilunar valves. Malalignment of great vessels with associated ventricular septal defects are visualized in high quality in malformed hearts, too. Embryonic chick hearts grown in shell-less culture were removed and fixed in dilated state at incubation day 9. Subsequent to critical point drying, the heart specimens were scanned within a Scanco Medical CT 80 workstation (SCANCO Medical, Zürich, Switzerland). For production of malformed hearts, chick embryos were treated on incubation day 2 (HH-stage 15) with Bis-diamine. Further details of the Micro-CT unit, methodology, 2-D and 3-D images, and fly-through movies with a digital journey in 3-D through normal and malformed hearts will be demonstrated during the meeting.

178. Genetic annotation of the developing heart

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When describing cardiac development, difficulties arise from naming the different anatomical compartments. This has several causes: 1. the common use of anatomical names by different researchers is too inconsistent to provide an adequate reference, 2. naming of compartments is historically based on the luminal configurations while the development of the chamber walls can be much more complicated and 3. embryonic cardiac compartments are not fully homologous to the similarly named adult compartments. To overcome these difficulties, we will pursue a novel approach to describe cardiac development, dubbed “genetic annotation”. In this approach the associations between expression domains of genes are used to discriminate different cardiac components during development. A limited number of marker genes can be used to genetically annotate cardiac compartments. This has been demonstrated previously, for example for the sinus node, which has a Cx30.2/Hcn4/Tbx3-high, Cx40/Cx43/Scn5a-low gene expression profile, and for the atrial wall myocardium for which the combination of Cx40 and Nppa can discriminate between appendage (Cx40+/Nppa+), primary (Cx40-/Nppa-) and dorsal (Cx40+/Nppa-) myocardium. The genetic annotation will be validated by mapping genes with a known role in cardiac development. We are currently collecting 3D expression patterns that will be mapped into a developmental reference series of 3D heart reconstructions. The resulting genetically annotated 3D reference models will also serve as a morphological framework to generate novel hypothesis on gene interactions, as positive gene interactions might be seen through gene co-localization (e.g. Cx40 is only expressed in Tbx5-positive myocardium) and negative interactions as complimentary gene expression patterns (e.g. Cx40 is not expressed in Tbx2-positive myocardium).

179. Optical pacing of the embryonic heart

Michael W. Jenkins* (1), Austin R. Duke (2), Shi Gu (1), Yong-Qiu Doughman (3), Hillel J. Chiel (4), Michiko Watanabe (3), E. Duco Jansen (2) and Andrew M. Rollins (1)
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Light has been used to noninvasively alter the excitability of both neural and cardiac tissue. Recently, pulsed laser light has been shown to be capable of eliciting action potentials in peripheral nerves and in cultured cardiomyocytes. Here, we demonstrate the first optical pacing of an intact heart ex ovo in a method we call optical pacing (OP). Embryos were cultured in Petri dishes in both shell-less cultures and New cultures. Pulsed 1.875 µm infrared laser light was employed to lock the heart rate to the pulse frequency of the laser. A laser Doppler velocimetry (LDV) signal was used to verify the pacing. At low radiant exposures, embryonic quail hearts were reliably paced without detectable damage to the tissue at stages 12 to 18. Compared to electrical pacing, OP does not require contact, has high spatial precision and avoids stimulation artifacts in electrode recordings. In the early embryo electrical pacing is invasive and difficult to achieve consistently and without tissue damage. In the future we will incorporate OP into our environmentally-controlled optical coherence tomography imaging chamber to study mechanotransduction (i.e. shear stress on the endocardium) by altering the heart rate of the embryo, thereby altering mechanical stress levels. OP will not only allow for a new class of experiments in developmental cardiology, but also may play a significant role in understanding cardiac electrophysiology, single-cell (cardiomyocyte) dynamics, and cardiac tissue engineering. Also, OP has the potential to pace an adult heart, which may be useful clinically. OP has the potential to become a robust technology for multiple medical and scientific applications in cardiology.

180. Imaging early development by High Resolution Episcopic Microscopy

Robert Wilson (1), Wolfgang Weninger (2) Shoumo Bhattacharya (3) and Tim Mohun* (1)
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The ability to analyse tissue and organ morphology (including cell proliferation and gene expression) in 3D is increasingly important for providing new insights into embryo development. Whilst technical difficulties in obtaining and manipulating 3D models have largely been resolved by advances in computing, obtaining high resolution data suitable for modelling remains a challenge. High Resolution Episcopic Microsocopy (HREM) is a relatively new episcopic (blockface) imaging technique that is ideally suited to computer modelling, providing 3D datasets that approach the resolution of conventional 2D histology. HREM relies upon the capture of sequential images of the embedded specimen block face during the sectioning process, rather than imaging individual sections that have been subject to histological staining. Inclusion of fluorescent dyes in the embedding medium provides a uniform background that effectively eliminates imaging of tissue below the surface of the sample block. HREM images retain their relative alignment without the need for reference markers and the resolution of each 3D dataset is determined by the combination of optical resolution and section thickness. 2D Image resolutions of less than 1 µm/pixel are readily achieved using plastic-embedded samples, with 3D isotropic resolutions of 1-3 µm. As part of a Wellcome Trust-sponsored initiative to establish an imaging pipeline for systematic analysis of mutant mouse embryos, we have used HREM to image normal mouse embryos at isotropic resolutions of 2-3 µm and present the data online (http://embryoimaging.org/). Each embryo has been sectioned in the transverse plane and data from any region of the embryo, including the heart, can be viewed at varying resolution, up to full scale. The same technique has been used to image the developing heart and the resulting models provide a unique resource for studying cardiac morphogenesis.

181. A new method for automated segmentation of the beating embryonic chick heart

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Functional analysis of beating embryonic hearts, for instance in chick embryos, is frequently conducted by generating real-time video recordings and subsequent computer aided manual examination of these recorded files with optimized software. During analysis images of the heart in end-systole and end-diastole are selected, predefined landmarks are located and traced manually in both heart phases. These landmarks allow calculation of cardiac function parameters such as shortening fraction or fractional area change. Paramaters such as the ejection fraction, stroke volume or cardiac output are estimated based on an elliptic or spherical volume model adapted to the manually identified landmark. Although some labs have implemented semi-automated analysis systems, it is desirable to develop new systems for a standardized and full-automated cardiac function analysis in large samples of embryos over significant periods of time and for all heart beats in a record. In this study, we present the first results of a new approach for full-automated segmentation of the beating embryonic chick heart which offers a time-continuous segmentation of image sequences of the heart during the cardiac cycle. Because the boundaries of the myocardium are barely visible in video recordings, it is impossible to segment the heart without using a-priori-knowledge. Thus, the embryonic heart is modeled as an active appearance model, consisting of a shape and texture model trained from various manual segmentations. The model parameters are iteratively adapted to video frames leading to a time-continuous segmentation of the beating heart that enables to extract cardiac function parameters.

182. Morphogenesis of cardiac chambers : integrating intrinsic cell behavior and external forces by computer modeling

Jean-François Le Garrec*(1), Chiara Ragni (1), Michel Kerszberg, Andrew Bangham (2), Enrico Coen (2), Margaret Buckingham (1) , Sigolène M. Meilhac (1)
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Mammalian heart morphogenesis involves looping and regionalized expansion of the initial tube, eventually leading to the fully functional four-chambered organ. Although many mutants defective for heart morphogenesis have been studied, how cell behavior and tissue mechanics combine to shape the heart remains poorly understood. We have previously analyzed the characteristics of clones of myocardial cells and shown that they grow with a preferential orientation, which is specific to each cardiac region. It is however difficult to disentangle how polarized growth, proliferation rate and external forces each contribute to the final shape. To understand the mechanics of the early myocardium and test the importance of oriented growth in shaping the heart, we combine image analysis and computer modelling. Quantitative analysis of confocal microscopy images allows us to assess the polarity of myocardial cells and their divisions as well as the relative rates of proliferation. We aim to integrate these experimental data, together with morphological observations and earlier results on patterns of clones, with the help of computer modeling. We present a Finite Element Analysis model, which simulates growth with regional differences in both rates and preferential directions, taking also into account external forces that might bear upon looping of the heart and chamber expansion. This model also permits simulation of the growth response to diffusing signals and thus should provide insight into how regionalization and polarity of the initial tube influence the final shape of the heart. Ultimately, it will be used to predict mutant shapes.

183. Automated recognition of the gene expression types in sections of embryonic mouse hearts.

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In cardiac embryology, research into the role of genes in development is of crucial importance. To study the function of genes, embryonic mouse hearts are fixated, sectioned and stained to visualise gene activity. This staining can either be done by using antibodies (ICC), or DNA probes (ISH). Gene expression can be described in terms of type and strength. The distribution of cells with a positive, above background, expression and the density of those cells result in the gene expression type. Currently the main gene expression types in use are: No expression, Scattered, Regional, and Ubiquitous. These expression types are mostly assigned by manual annotation, which is not only time consuming but also subjective. These issues can be resolved by automated recognition of the gene expression types. In order to create an automatic method, a survey has been performed to determine the definitions of gene expression types in use in the field of cardiac development. The results of this first survey were used to create a second survey in which experts were asked to annotate expression types of mouse-heart sections. These results, and the determination of a number of image features in the same heart sections, will serve as a learning-set to create classification rules. These rules then form the foundation for the automated recognition of gene expression types. In a pilot experiment based on virtual sections, which was also based on classification rules, we have shown to be able to automatically annotate gene expression patterns in embryonic mouse heart sections.

Session C - Cardiac Growth

184. Knockdown of the orphan G protein-coupled receptor 126 reduces heart rate and influences ventricular morphogenesis in zebrafish

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Gpr126 is an orphan G protein-coupled receptor (GPCR). The lack of knowledge regarding its natural or synthetic ligands makes it difficult to determine its biological function. Based on an mRNA expression screen we have identified gpr126 as a gene that is transiently expressed during embryonic rat heart development. gpr126 mRNA is highest expressed in the heart from E13.5 to E17.5 and declines thereafter. RT-PCR showed that gpr126 mRNA is also expressed in the zebrafish embryo. gpr126 mRNA is absent in the early zebrafish embryo (1 and 9 hours post fertilization (hpf)) and its expression starts around 24 hpf. By in situ hybridization at 44 hpf, we observed expression of gpr126 mRNA in the heart ventricle and atrium. To investigate how gpr126 may function in heart development we injected translation- and splice-inhibiting morpholino antisense oligonucleotides (MOatg and MOsplice respectively) to knockdown gpr126 in zebrafish embryos. MOsplice resulted in deletion of exon 13 which was readily detectable by RT-PCR at 80h hpf. Following injection of either MOatg or MOsplice, cmcl2-GFP transgenic embryos exhibiting heart specific fluorescence were collected from different stages and screened for morphological and functional defects. gpr126 knockdown resulted in a significantly reduced heart rate and blood accumulation between the sinus venosus and the atrium, which was first observed around 34 hpf. Furthermore, at 56 hpf gpr126 knockdown embryos exhibited a dysmorphic ventricle and apparent cardiac edema with otherwise normal overall morphology. In conclusion, this study reveals an essential role for gpr126 in cardiac development.

185. VEGF Gene Therapy Revives Coronary Artery Development in the Conotruncal Banded Chick Embryo

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Constriction of the conotruncus (CT) during early embryonic development alters the migration of mesenchymal tissue into the heart causing failure of absorption in bulbus arteriosus, misalignment of both great vessels, and reduction of coronary vascularization. We hypothesized that the non-viral transfection of VEGF would have a beneficial role in vascular repair and proliferation following mechanical injury to the developing heart. The CT of a stage 21 (3½-d) chick embryo was tied with an overhand knot using 10-0 nylon suture. Hypoxia-responsive pRTP801-VEGF plasmid was complexed with the reducible cationic polymer, CBA-DAH-R (ABP), in HEPES buffer (5 ng/5 mL), and infused into the compact layer of CT-banded heart at stage 29 (6-d). Normal embryos did not undergo operation. At stage 36 (10-d), India ink in PBS (1:5) was injected into the left ventricle and subsequently filled the coronary arteries. The heart was fixed in diastole with 1 mg/Kg diltiazem in 4% paraformaldahyde and imaged under a stereophotomicroscope. Morphometric analysis was performed to determine the maximum coronary artery distance, and ventricular vertical length. Data are presented as mean±SEM ($n \geq 8$), and analyzed by ANOVA. Ventricular lengths showed no statistical difference ($p>0.05$) among normal, CT-banded, and CT-banded with pRTP801-VEGF/ABP hearts. Coronary artery length indexed by ventricular length was similar between CT-banded VEGF-transfected hearts (0.47±0.04, $p>0.05$) and normal embryos (0.50±0.02). This stands in contrast to a marked decrease in CT-banded hearts (0.34±0.05, $p<0.05$) when compared to normal control. Coronary arteries in CT-banded hearts with pRTP801-VEGF/ABP treatment undergo proportionate vascularization and penetration comparable to normal development.

186. Nephronectin is required to restrict the differentiation

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The extracellular matrix plays an important role in morphogenesis, tissue repair and disease. Here, we show that the gene npnt encoding the extracellular matrix protein Nephronectin is transiently expressed in the myocardium of the developing zebrafish heart. To further explore the role of nephronectin in cardiac development, we knocked down npnt expression through translation and splice inhibitory morpholinos. Nephronectin morphants exhibited cardiac edema and severe defects in cardiac looping that could be rescued by injection of npnt mRNA. Moreover, npnt knockdown caused expansion of the cardiac jelly, which is similar to defects observed in a tbx2 overexpression mouse model. Interestingly, expression of has2 and its upstream regulators tbx2 and bmp4 that usually are restricted to the atrio-ventricular boundary was expanded in npnt morphants. Detailed morphological analyses suggested further that the knockdown of npnt caused an extension of the atrio-ventricular canal. This was in accordance with an increased number of cuboidal-shaped and Alcam-positive differentiated endocardial cells as well as an expanded expression of notch1b. Our results identify nephronectin as a new regulator of cardiac development playing a crucial role in the differentiation of the atrio-ventricular canal of the zebrafish heart.

187. Three-Dimensional and Molecular Analysis of the Venous Pole of the Developing Human Heart

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Various congenital malformations, as well as many abnormal rhythms, originate from the venous pole of the heart. Due to rapid changes during morphogenesis, lack of molecular and lineage data, along with miscommunications owing to difficulties in forming a mental image of the complex morphogenetic changes in the developing heart, the development of this region in human is difficult to grasp. To get more insight into the development of the different types of myocardium forming the venous pole of the heart, we perfermed an immunohistochemical analysis on serial sections of early human embryos and prepaired the three-dimensional models. As in the mouse, the systemic venous myocardium expresses the transcription factor TBX18, while the pulmonary venous myocardium expresses NKX2-5; however, in contrast to mouse, both structures express ISL1. In human embryos, prior to appearance of venous valves, a systemic venous sinus is identified upstream from the atrial chambers, albeit initially with non-myocardial walls. From the outset, as in the mouse, the pulmonary vein empties to a chamber with atrial, rather than systemic venous, characteristics. When compared to the mouse, the vestibular spine is a more prominent structure. The remarkable similarities in gene expression in the distinctive types of myocardium surrounding the systemic and pulmonary venous tributaries in man and mouse permit extrapolation of the conclusions drawn from transgenic and lineage studies in the mouse to the human, showing that the systemic and pulmonary venous myocardial sleeves are derived from distinct developmental lineages, and lends no support to the notion that the pulmonary myocardium originates from the systemic venous sinus.

188. Activation of various stress response pathways in cardiomyocytes during embryonic heart regeneration

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Utilizing a heart conditional Knock-out of the X-linked Holocytochrome c synthase (Hccs) gene in mice we previously reported a remarkable regenerative potential of the embryonic heart in response to tissue mosaicism for mitochondrial dysfunction. Despite the functional loss of 50% of Hccs deficient cardiomyocytes at midgestation, compensatory hyperproliferation of healthy myocardial cells ensures the generation of a functional heart by birth. To investigate the underlying molecular and cellular mechanisms we performed RNA expression analyses in regenerating hearts at various stages of development. These data revealed activation of the endoplasmic reticulum (ER) stress and unfolded protein response (UPR), as characteristic genes involved in translational control as well as amino acid metabolism were significantly upregulated (e.g. Atf-4, Atf-5, Asns, Slc7a3). Induction of p21/Cip1 and p27/Kip1 furthermore suggests activation of cell cycle inhibitors in response to cell stress. Additionally, expression of several proapoptotic genes was increased in regenerating hearts (e.g. Gadd-153, Noxa, Edr2a, Chac-1) which might be attributed to Hccs deficient cardiomyocytes as these cells supposedly experience excessive stress. Given that there is no evidence for apoptosis in regenerating hearts this argues for additional cell protective mechanisms. Indeed, expression of heat shock protein (Hsp) 60 as well as the reactive oxygen species (ROS) converting enzyme superoxide dismutase (Sod)-2 and gap junction protein connexin 43 was specifically induced in Hccs deficient cells. Taken together, our data reveal activation of various cell stress and survival mechanisms in cardiomyocytes during embryonic heart regeneration which putatively contribute to the generation of functional hearts by birth.

189. Cyclin D2 is a GATA-4 cofactor involved in cardiac growth

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For decades, postnatal cardiomyocytes have been considered as terminally differentiated cells that lost their ability to proliferate; thus, the mature mammalian heart has very limited regenerative capacity. Persistent cardiac stress causes myocyte loss that often leads to heart failure. Recent studies uncovered limited proliferative capacity of cardiomyocytes albeit the underlying mechanism is poorly understood. Transcription factor GATA-4 is a key regulator of cardiomyocyte growth and differentiation. Lack of GATA-4 impairs adaptive growth response and upregulated expression of GATA-4 is sufficient to induce myocyte hypertrophy. Search for GATA-4 targets/effectors revealed cyclin D2 (CD2), a member of the D-type cyclins (D1, D2, and D3) that play a vital role in differentiation and cell cycle regulation, as a direct transcriptional target and an effector of GATA-4. Biochemical analysis revealed that CD2 enhances GATA-4 activation of target promoters. Our results explain, in part, why upregulation of either protein is sufficient to turn on myocyte growth. To check which action of GATA-4 is mediated by CD2, we developed lines of transgenic mice with myocardial specific expression of CD2. These mice had normal cardiac functions aside from evidenced increase in left ventricular mass. Immunohistochemical staining revealed increased expression of two proliferation markers. When crossed with GATA-4+/- mice, myocardial CD2 was able to rescue, at least partially, cardiac growth of GATA-4 heterozygotes. These data suggest that myocardial CD2 has beneficial effects on the heart and will shed more light on the role of cell cycle proteins, particularly cyclin Ds, in the expansion of differentiated cells and provide novel avenues for heart failure therapy.

190. Follistatin-like 3 contributes to cardiac hypertrophy and remodelling

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The balance between activins and their inhibitors regulates cardiac growth and the response of the heart to stress. Follistatin-like 3 (Fstl3) is a potent inhibitor of activin signalling that antagonises the cardioprotective role of activin A after myocardial infarction. We have previously reported that Fstl3 expression is elevated in patients with heart failure and upregulated by hypertrophic stimuli. However its role in cardiac remodeling is largely unknown. Here we show that in hypertrophic hearts Fstl3 is mainly produced by cardiomyocytes. Fstl3 is sufficient to induce cardiomyocyte hypertrophy in culture, as demonstrated both by increased cell surface area and protein synthesis. Cardiomyocyte-specific Fstl3 knockout (KO) mice show improved cardiac function following pressure overload and reduced expression of heart failure markers compared to wild type mice. In addition, Fstl3 KO mice develop less fibrosis and express lower levels of fibrosis markers than wild type mice. Yeast two hybrid experiments suggest that Fstl3 acts at least in part through binding to proteins of the extracellular matrix (ECM) as well as pro-fibrotic factors, including connective tissue growth factor. In summary, our data suggest that Fstl3 is secreted by cardiomyocytes in the failing myocardium and plays a role in cardiac hypertrophy and fibrosis through autocrine and paracrine mechanisms. Fstl3 may therefore represent a novel therapeutic target for the treatment of heart failure.

191. Ordered assembly of the adhesive and electrochemical connections within newly formed intercalated disks

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The functional coupling of immature cardiac myocytes during development requires the establishment of new electromechanical connections between neighboring cells to form a functional syncitium. The primary site of coupling occurs at the terminal ends of the myofibrils in direct cell-cell contacts termed intercalated disks (IDs). The ID is composed of adhesive elements, including fascia adherens and desmosomes, and electrochemical communications, including ion channels and gap junctions. The integrity of the disk is essential for normal cardiac development and function, but how its diverse elements are assembled into a fully integrated structure is not well understood. In this study, we examined the assembly and organization of cardiac intercalated disks in primary cultures of adult rat cardiac myocytes. As the cells remodeled and made contact with their neighbors, transmembrane adhesion proteins, including N-cadherin and desmocollin, localized along the line of apposition. After development of the adhesion plaque, Cx43 gap junctions and ankyrin-G, an essential cytoskeletal component of the voltage-gated sodium channel, were secondarily recruited to the new intercalated disks, selectively localizing to membrane domains involved in cell-cell contacts. The precise order of the assembly process suggests that there are specific scaffolding requirements for the integration of the mechanical and electrochemical elements of the disk. Defining these relationships will be important for promoting the functional integration of immature cardiomyocytes into injured myocardium during cellular cardiomyoplasty and for treating the mechanical dysfunction and cardiac arrhythmias that accompany alterations of intercalated disk architecture during heart failure.

192. Heterogeneity of vessels within the mouse myocardium identified by lymphatic markers

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Introduction: The arterial supply and venous drainage of the 4-chambered heart are well characterized. The lymphatic drainage of the heart has received less attention. Our previous analysis indicated that vessels and cells within the developing myocardium express a subset of lymphatic markers but may not be lymphatics. To deduce their final distribution and fate, we extended our analysis to the adult mouse heart. Materials and Methods: Serial sections of adult mouse hearts were immunostained for (1) PECAM, to identify all endothelial cells and markers of lymphatic endothelial cells (2) Prox1, (3) LYVE-1, (4) Podoplanin and (5) VEGFR3. Results: As expected, we found a lymphatic system within the epicardium co-expressing Prox-1, LYVE-1, Podoplanin and VEGFR3. However, even in the adult, vessels in the myocardium stained for PECAM, LYVE-1 and VEGFR3 but not Prox1 or podoplanin. These vessels also immunostained for ephrinB4, a venous endothelial cell marker. Serial sections revealed that these vessels contained blood cells and were continuous with the ventricular lumen. Conclusions/Discussion: In combination with data regarding lymphatic markers in developing hearts, our findings suggest that lymphatic markers identify bona fide epicardial lymphatics. Surprisingly such lymphatics were largely absent from the myocardium. Myocardial vessels expressing a subset of the lymphatic markers and having venous characteristics connect directly to the lumen of the heart. These may have arisen from invaginations of the endocardium and may be Thebesian vessels that are involved in the regulation of fluid balance in the heart.

Session C - Conduction System

193. Origins of Cardiac Pacemaking Cells in Avians

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For abstract, see in oral presentation section 77

194. The role of Tbx3 and Nkx2-5 in the adult atrioventricular conduction system

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For abstract, see in oral presentation section page 78

195. Delayed atrioventricular conduction in adrenergic-deficient embryonic mouse hearts: Uncovering a role for retinoic acid

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For abstract, see in oral presentation section page 79

196. Development and Electrical Activation of Sinus Venosus Myocardium in the Avian Embryo

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Purpose: The sinus venosus myocardium, including the sinoatrial node (SAN), is recruited to the primary heart tube during development. Podoplanin is expressed in this myocardial area and can interact with RhoA. Additionally, alteration of RhoA expression leads to defective conduction. We aim to elucidate the development of sinus venosus myocardium, including the SAN, by studying expression patterns of RhoA in relation to other differentiation markers and by studying electrical activation patterns during development. Methods: Expression of RhoA, cTnI and Nkx2.5, Isl-1 and Tbx18, and cation channel HCN4, were examined in sequential stages in chick. Electrical activation patterns were studied using ex-ovo local micro-electrode recordings and optical mapping. Results: Sinus venosus myocardium is cTnI and HCN4 positive but Nkx2.5 negative and is complemented by distinct Isl-1 and Tbx18 patterns. Initial myocardium-wide expression of RhoA becomes restricted to the right-sided sinus venosus myocardium, comprising the SAN. This shift coincides with the persistence of RhoA, Tbx18 and HCN4 expression and absence of Nkx2.5 in the definitive SAN. Electrophysiological measurements reveal initial capacity of both left and right sinus venosus myocardium to show the first electrical activity that in time shifts to a right-sided dominance. Conclusion: Our results suggest a role for RhoA in conduction system development. Additionally, given the observed electrical potential of the sinus venosus myocardium, we propose an initial sinus venosus wide capacity to generate pacemaker signals, becoming confined to the definitive SAN. Lack of differentiation towards a chamber phenotype would explain ectopic pacemaker foci.

197. Mechanical loading is required for early conduction system development

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Increased hemodynamic loading in vivo has been shown to accelerate normal cardiac morphogenesis as well as conduction system maturation in the chick embryos. To test the effect of hemodynamic unloading, we employed whole heart culture of chick and mouse embryonic hearts at different developmental stages and analyzed ventricular activation sequence and morphology. Epicardial activation patterns were assessed by optical mapping. In control, freshly isolated hearts of Cx40:GFP heterozygous mouse embryos between E10.5 and E11.5 there are two dominant preferential activation pathways: the first utilizes the primary ring, while the more mature one employs the His bundle and its branches for ventricular activation from apex to base. In experimental hearts cultured for 24 hours we observed a regression in normal developmental timeline with appearance of ventricular activation patterns from base to apex. Total ventricular activation time was also prolonged in culture. Even more pronounced effect was observed in chick embryonic hearts isolated at E3 and E4, where dedifferentiation of the ventricular activation sequence could be rescued by artificial ventricular loading with a droplet of silicon oil imposing passive stretch. Morphogenesis of hearts in culture was arrested at the time of explantation. We conclude that appropriate mechanical loading is a prerequisite for ventricular conduction system maturation during the early phases of development in both birds and mammals. Supported by MFM VZ 0021620806, AV0Z50450515 and AV0Z50110509, and GACR 304/08/0615.

198. Cardiac conduction system anomalies associated to ion channels remodeling during development in a transgenic model of Long QT-Syndrome.

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Long QT syndrome (LQTS) is characterised by an increase of the cardiac action potential duration and it has been related to cases of sudden death in newborns. LQTS has been linked to mutations in distinct ion-channel-coding genes, such as KCNQ1 and SCN5A. The mouse transgenic line α-MHC-KvLQT1-iso2-T7 shares phenotypic characteristics with the LQTS. These mice display molecular and morphological characteristic of hypertrophic cardiomyopathy (HCM). Because hypertrophic process in the heart has been previously related with sodium channel remodeling, we analyzed sodium channel gene expression in LQTS mouse model and show that Scn5a and Scn1b up-regulation occurs at embryonic stage (i.e. E13.5), before the HCM phenotype is established. Moreover, optical mapping analyses evidenced a delayed electric impulse propagation thought the cardiac conduction system at adult and fetal stages. Intercrossing these transgenic mice with Cx40-GFP mice, we found an increase of spread out fibers in the apical part of the Left Bundle Branch (LBB) as well as changes in the number of the fibers that achieve the free LV wall from LBB. Similar results were observed for Right Bundle Brunch (RBB) in Cx40-GFP+/- α-MHC-KvLQT1-iso2-T7 mice respect to Cx40-GFP+/- littermates. These morphological alterations in cardiac conduction system were already detected at fetal stages. In summary, we provide evidence for the first time that cardiac conduction system malformations are associated to ion channel remodeling during heart development. This study may open new ways to better understand clinical pathologies such as cardiac congenital anomalies, arrhythmias and perinatal sudden death.

199. Trabecular fate mapping using a Cx40-CreERT2 mouse line

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The rapid propagation of electrical activity in the ventricles is executed by the ventricular conduction system (VCS), composed of the His bundle, left and right bundle branches and the peripheral Purkinje fiber network. In adult hearts of Cx40GFP/+ mice, the GFP reporter gene is specifically expressed throughout the VCS. During embryonic development, GFP is expressed in trabecular cells, which are considered to be precursors of conductive myocytes. The aim of our study is to define the fate of trabeculae during normal heart development. Using a prospective cellular lineage analysis we investigated whether trabeculae give rise to contractile cardiomyocytes as well as Purkinje fibers using a novel transgenic mouse line (Cx40KI-CreERT2-RFP) expressing the tamoxifen-inducible Cre recombinase specifically in the trabeculae and developing VCS. The RFP reporter gene (CreERT2-RFP transgene) is expressed in the same tissues as described for the endogenous Cx40 gene during embryonic development including endothelial cells, muscle and heart. We next carried out prospective Cre lineage mapping by crossing the Cx40Cre/+ with R26R mice. After injection of tamoxifen, β-galactosidase expression is detected in Cx40 expressing cells and their derivatives. Pregnant females were injected with tamoxifen at different timepoints, in order to monitor different steps of trabecular development and define the time window in which the VCS differentiates. Our preliminary results reveal progressive restriction of myocardial fate to the VCS, and support the existence of distinct developmental phases during establishment of the VCS. In addition, these mice will be useful for conditional gene expression and inactivation in the definitive VCS.

200. Cell death in the atrioventricular canal myocardium determines ventricular activation patterns

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Based on our previous study showing accelerated conversion of ventricular activation sequence during hypoxic incubation correlating with increased apoptosis in the atrioventricular canal, we hypothesized that this slight increase in apoptosis could lead to an accelerated pruning of myocardial atrioventricular connections, which would manifest as earlier appearance of mature apex-to-base activation patterns. To test this hypothesis, we blocked apoptosis using an established caspase inhibitor in chick embryonic heart and analyzed the effects of this treatment on cell death and ventricular activation patterns. ED4 chick embryonic hearts were treated with the peptide Caspase inhibitors zVAD-fmk by intrapericardial injection in ovo. Spontaneously beating embryonic hearts isolated at ED8 were stained with voltage-sensitive dye C with Ultima L high-speed camera at 1 kHz. Amount°Di-4-ANEPPS and imaged at 37 of apoptotic cells was analyzed at ED 7 by whole mount LysoTracker Red staining and confocal microscopy. Hearts of embryos treated with zVAD showed a significantly increased proportion of immature (base to apex) activation patterns at ED8, including ventricular activation originating from right atrioventricular junction, a pattern never observed in control hearts. The number of apoptotic cells in atrioventricular canal myocardium was decreased at ED7 in the treated hearts. Apoptosis in the atrioventricular canal myocardium is thus an important contributor to ventricular activation pattern. Its inhibition at critical stages can lead to persistence of accessory atrioventricular connections, which form a morphological substrate for ventricular pre-excitation. Supported by project of MSMT VZ 0021620806, LC 06061, and by grant of AS CR AV0Z50450515

201. Electrophysiological Mapping of Embryonic Mouse Hearts: Mechanisms of Dominant Pacemaker Switch during Early Cardiogenesis

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Introduction: Pacemaker activity originated from the left sinus venosus (LSV) area of embryonic mouse hearts at 8.5 days post coitus (E8.5). This dominant pacemaker activity switches to the right-sided sinoatrial node (SAN) with further cardiac development. The underlying molecular mechanism of pacemaker location switch during cardiogenesis remains poorly understood. An electrophysiological (EP) mapping method of embryonic hearts without any non-physiological influences from dye-loading processes will yield most reliable information regarding the true mechanism underlying pacemaker location switch. Methods: EP recordings with multi-electrode array (MEA) on acutely isolated mice heart preparations at E8.5-E12.5 were performed. Results: Using pharmacological agents and EP recordings, we found that intracellular Ca2+-mediated mechanisms (including Ryanodine receptors and Na+/Ca2+ exchangers) developed early and dominated the control of automaticity in LSV of E8.5 hearts. With pharmacological blockers, an immature pacemaker located at RSV could be demonstrated at E8.5 after complete arrest of the LSV pacemaker. With further embryonic development, clear SAN signals could be recorded in E11.5-12.5 hearts. Importantly, a mature form of dominant automaticity, involving both sarcolemmal ion channels and intracellular Ca2+ handling apparatus, evolved in SAN upon further differentiation at E12.5. This mechanistic maturation of automaticity appeared to accompany the location switch of dominant pacemaker regions during early cardiogenesis from E8.5 to E12.5. Loss of left-sided dominance of automaticity coincided with down-regulation of HCN4 channels, suggesting inhibitory signals exist at LSV area during development. Conclusions: Intracellular Ca2+ handling apparatus developed early in LSV at E8.5 for controlling automaticity. However, an immature and non-dominant pacemaker could be demonstrated in RSV at E8.5. Voltage-dependent HCN and Ca2+ channels evolve at SAN to play increasing roles in pulse generation, which permits faster responses to rising metabolic demands. This mechanistic maturation confers the pacemaker location switch.

202. Myocardial specific deletion of Tbx2 leads to ventricular preexcitation

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Atrioventricular (AV) reentrant arrhythmias are caused by accessory myocardial pathways between the atria and ventricles that cross the annulus fibrosus. In the normal pre- and early postnatal heart, however, these AV myocardial connections are common, yet AV arrhythmias are rare, indicating that the basis of the formation of functional arrhythmogenic pathways is not understood. Transcription factor Tbx2 is expressed in the AV myocardium and endocardial cushions, and is critical for AV canal formation. Here, we show that global and myocardium-specific inactivation of Tbx2 causes formation of an accessory pathway in the AV junction and malformation of the annulus fibrosus. These pathways cause ventricular preexcitation, and, consistently, ectopically express high conductance gap-junctions Cx40 and Cx43 and cardiac sodium channel Scn5a. A fraction of fetal Tbx2-mutants did not display arrhythmias despite the presence of these pathways, suggesting a Tbx2-independent mechanism that prevents functionality at that stage. AV canal-specific proteins including Cx30.2, which decelerates the impulse in the adult AV node, were found to be expressed in the AV canal myocardium and maintained in Tbx2-deficient fetuses in variable patterns. The role of Cx30.2 in the functionality of the accessory pathway of Tbx2-mutants was investigated. Our data indicate three processes preventing the formation of functional accessory pathways: 1) correct patterning and morphogenesis of the AV canal myocardium to guide annulus fibrosus formation, 2) repression of the working myocardial gene program, and 3) loss of the AV canal specific gene program. Myocardial specific loss of Tbx2 affects these processes leading to the formation of functional accessory bundles.

203. Atrial Myocyte Heterogeneity in Connexin Expression and Action Potential Conduction during Chick Cardiac Development.

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The cardiac conduction system (CCS) consists of several distinct subcomponents, including the pacemaking SA node, internodal tract (INT), AV node, and Purkinje fibers. While differentiation of Purkinje fibers from ventricular myocytes has extensively been studied, little is know about mechanisms that induce or pattern other CCS components. For example, mounting physiological evidence indicates the presence of fast INT pathways in the atrium, however, there is little evidence indicating the presence of a morphologically or molecularly distinct cell population that comprises the INT. Here we report optical mapping data which demonstrate that, during heart looping, action potentials propagate across the atria in a non uniform pattern, instead showing tracts of cells with increased conduction velocity. Coinciding with these preferential conduction tracts, embryonic atria display molecular heterogeneity in their expression of several connexins including fast conducting Cx40 and slow conducting Cx45. Collectively these data suggest that a molecularly distinct subpopulation of cx40 positive atrial myocytes is present during INT tract development in chick. Supported in part by grants from the NIH-NHLBI.

Session C - Genetics & Cardiovascular Malformations

204. Dysregulation of the PDGFRA gene causes inflow tract anomalies including TAPVR: Integrating evidence from human genetics and model organisms

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For abstract, see in oral presentation section page 53

205. Effects of conditional deletion of hypoxia-inducible factor (hif) and hypoxic stress on mouse heart morphogenesis

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For abstract, see in oral presentation section page 54

206. p53-related p63 transcription factor is essential for cardiac morphogenesis

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For abstract, see in oral presentation section page 55

207. Copy Number variations (CNV) in left sided congenital heart disease

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208. The role of FKbp12-notch1 mediated regulation in ventricular trabeculation and compaction

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For abstract, see in oral presentation section page 57

209. MCTP2 is a novel gene causing aortic coarctation

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210. Loss of Akt1 causes intra-uterine stress leading to heart defects

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In a C57/B6 genetic background, the majority of Akt1-deficient mice display post-natal mortality. Histological analysis of Akt1-deficient embryos and newborns revealed congenital heart defects, decreased cell proliferation and vascular intensity. Echocardiographic study of young Akt1-deficient mice indicated decreased heart function. Loss of Akt1 caused substantial activation of the p38MAPK pathway. Breeding the Akt1-deficient mice to mice that were heterozygous for a null p38α allele partially rescued the congenital heart defects, significantly decreased post-natal mortality, and restored normal patterns of cardiomyocyte proliferation. Our study suggests that Akt1 is essential for heart development and function, in part, through suppression of p38MAPK activation.

211. Regulation of Hand1 by Akt/PKB is involved in pathological cardiac remodeling

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Aberrant activation of Akt signaling causes pathological cardiac remodeling, such as hypertrophy and heart enlargement leading to heart failure. However, the downstream players of Akt signaling remain elusive. Here, we reported that Hand1, a basic helix-loop-helix (bHLH) transcription factor essential for heart development, could be a downstream target of Akt signaling involved in pathological heart remodeling. A well-conserved Akt substrate consensus motif has been identified in Hand1 proteins from different species suggesting Hand1 as a putative Akt substrate. Using biochemical analysis, we demonstrated that Hand1 was phosphorylated by Akt at threonine 107(T107) and serine 109(S109) in the basic-helix domain. Phosphorylation of Hand1 regulates its transcriptional activation of luciferase reporter genes, DNA binding ability and cellular localization. Furthermore, transgenic mice with over-expression of Hand1 mutants (to mimic or to abolish phosphorylation) in cardiomyocytes developed pathological cardiac remodeling. This study provides novel insights into Akt signaling in cardiac remodeling and Hand1 regulation in post-natal heart function.

212. 14-3-3 epsilon is essential for heart morphogenesis and involved in human left ventricular noncompaction

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Left ventricular noncompaction (LVNC) is a human cardiomyopathy resulting from defective ventricular morphogenesis, a process regulated by cardiomyocyte proliferation. 14-3-3ε (encoded by Ywhae) is an adapter protein involved in cell proliferation, and Miller-Dieker syndrome, a severe human lissencephaly. We hypothesized that Ywhae modulates cardiogenesis because of its characteristic embryonic expression pattern. Ywhae homozygote mutant mouse hearts presented specific features of LVNC, and a selective defect of the ventricular compact myocardium. Ywhae is essential for cardiac proliferation, maintaining low levels of the cell cycle inhibitor p27Kip1 by promoting its ubiquitination. Because of Ywhae dosage effects in the mouse heart, we screened seventy seven LVNC patients for YWHAE mutations. We identified two probands with a novel heterozygous substitution in a CCAAT/enhancer binding protein site of YWHAE which represses its promoter activity. This work uncovers a novel role of Ywhae in mammalian heart development, and provides the first evidence implicating YWHAE in cardiomyopathies.

213. Periconceptional nutrient intakes and risks of conotruncal heart defects

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We investigated associations between conotruncal heart defects and periconceptional multi-vitamin use and intakes of dietary nutrients. Data derived from a population-based case-control study of fetuses and liveborn infants among almost a million California births between July 1999 and June 2004. Cases included 140 with d-transposition of great arteries (dTGA), and 163 with tetralogy of Fallot (TOF). Total number of controls was 698. Use of vitamins was elicited by questionnaire for the periconceptional period. Dietary nutrient intake was elicited by a well-known food frequency questionnaire. Results showed that the odds ratio for dTGA associated with supplemental multivitamin use was 1.0 (95% confidence interval, 0.7-1.5) and for TOF was 0.9 (0.6-1.3). We observed increased risks associated with lower dietary intakes of linoleic acid, total carbohydrate, and fructose for dTGA. Among women who did not use vitamin supplements periconceptionally, we found increased risks of dTGA associated with lower dietary intake of several micronutrients -- folate, niacin, riboflavin, and vitamins B12, A, and E, even after simultaneous adjustment for other studied nutrients. We did not find similar associations among mothers who delivered infants with TOF. Analytical consideration of several potential confounders did not reveal alternative interpretations of the results. Evidence continues to accumulate to show that nutrients, particularly folate, influence risks of structural birth defects. Our results extend observations that other nutrients may also be important in heart development.

214. Novel and common chromosomal imbalances associated with congenital heart defects.

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In an effort to identify new genetic factors associated with congenital heart defects (CHD) we have studied 328 prenatal and postnatal DNA samples of patients with syndromic and isolated CHD. We selected a subset of 63 patients on which we typed 660.000 SNPs for intensity and genotype using the Illumina 660W beadchip. 20 of the 63 patients were syndromic and 43 suffered of an isolated CHD. As a result of this study we detected 6 chromosomal abnormalities that are the putative cause of the observed CHD: 1) 2 chromosomal abnormalities of chromosome 22; an atypical duplication of 22q11.2 associated with a ventricular septal defect (VSD) and cleft palate, and an atypical deletion of the same region associated with an isolated Tetralogy of Fallot (TOF). This region does not contain genes known to be implicated in DiGeorge syndrome or with heart development. 2) A duplication of the 17q12 region associated with a TOF. This region not previously contains the LHX1 gene that is known to be involved in mesoderm and neural development. 3) A patient with an apparently equilibrated reciprocal translocation between chromosome 2 and 7 with an isolated TOF was found to have deletions on both chromosomes affecting a series of novel candidate genes. 4) Finally, we also detected a deletion of 11q24-25 which identifies the patient as suffering from Jacobsen syndrome (CIV and developmental delay); and a 17q21.31 deletion that identifies the patient as suffering of the 17q21.31 syndrome (CIV and multiple other abnormalities).

215. Identification Of An Essential Gene For Cardiovascular Development From Studies On The L11jus8 Mutant Mouse

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The l11Jus8 recessive mutation was isolated from a balancer chromosome mutagenesis screen on mouse chromosome 11. Macroscopically, l11Jus8 mutants exhibit thoracic haemorrhage, impaired yolk sac vascular maintenance, and mid-gestation lethality. Histological analysis revealed loss of atrial and outflow tract myocardial integrity, potential apoptosis of atrial cardiomyocytes, and excessive blood pooling in l11Jus8 mutant heart chambers. Thin ventricular myocardium and reduced trabeculation are exhibited in ~30% of l11Jus8 mutants. Immunohistochemical analyses demonstrate progressive reduction in mutant yolk sac vascular diameter whilst embryonic vasculature appears unaffected. The nature and temporal occurrence of abnormalities in l11Jus8 mutants implies a primary cardiac defect with secondary yolk sac vascular defects. Ultrasound will be utilised to evaluate cardiac function throughout development in mutant embryos. The gene containing the l11Jus8 mutation is unknown. Meiotic mapping has refined the l11Jus8 mutation to a ~2.3Mb region including ~45 predicted transcripts. Exonic and splice junction genomic DNA of 20 high priority genes associated with cardiovascular development or embryonic lethality have been sequenced, although the l11Jus8 causative mutation remains to be discovered. Future work will employ genome capture and high-throughput sequencing to identify the l11Jus8 causative mutation. As no similar phenotypes have been attributed to mutations of genes in the l11Jus8 candidate region, positional cloning of the l11Jus8 mutation will provide a new link between a gene in the candidate region and cardiac development. Study of the atrial abnormalities in l11Jus8 mutants will allow a better understanding of the general process of atrial development, which is poorly characterised at present.

216. Great vessel development requires biallelic expression of Chd7 and Tbx1 in pharyngeal ectoderm in mice

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CHARGE syndrome is characterised by coloboma, heart defects including Tetralogy of Fallot, choanal atresia, retarded growth and development, genital and ear abnormalities. Deletion or mutation of chromodomain protein CHD7 is the major cause of this syndrome. Di George syndrome is frequently associated with a deletion of 22q11.2 and haploinsufficiency of T-box transcription factor TBX1 causes a number of symptoms including great vessel defects particularly of the outflow tract and aortic arch. Such cardiovascular malformations arise from defective remodelling of the pharyngeal arch arteries (PAA). The significant phenotypic overlap of these disorders and the fact that Chd7 is the only gene other than Tbx1 reported to affect 4th PAA development by haploinsufficiency, led us to investigate the embryological relationship between the two syndromes using mouse models. Hypo/aplasia was observed in both Tbx1 and Chd7 heterozygote mice at E10.5 with resulting interruption of the aortic arch observed at later stages. Double heterozygous mice displayed a synergistic interaction for this phenotype. Restoration of Chd7 in the neural crest or mesoderm did not rescue PAA morphogenesis, however dizygous expression of Chd7 and Tbx1 in the pharyngeal ectoderm was required for normal PAA development.

217. Genetic analysis of the role of Nodal-related southpaw in the regulation of cardiac asymmetry

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In all vertebrates, the heart is the first organ to establish left-right asymmetry during development, and defects in L/R asymmetry may result in congenital cardiac defects. During zebrafish embryonic development, asymmetric gene expression drives leftward displacement of the linear heart tube, and is followed by heart looping, which is crucial for proper alignment of the cardiac segments and subsequent heart function. The first known asymmetrically expressed gene in zebrafish is southpaw (spaw), a Nodal-related gene whose expression is propagated from posterior to anterior in the left LPM during somitogenesis. In a forward genetic screen we have identified a zebrafish mutant carrying a mutation in the spaw gene. Spaw mutants display a failure of cardiac displacement, and subsequent randomisation of and/or incomplete cardiac looping. While it is clear that spaw is required to regulate initial cardiac asymmetry, it is unclear how asymmetric spaw expression translates into asymmetric cardiac morphogenesis. To determine the downstream factors required for translating the spaw signal into asymmetric cardiac morphogenesis, we have performed microarrays to analyse differential gene expression between wild type and spaw mutant embryos at 10, 15 and 23 somite stages, prior to initiation of cardiac asymmetry. Furthermore, in situ analyses reveal that Spaw function is required to propagate asymmetric spaw expression in the left LPM. To determine the mechanism by which the spaw signal is asymmetrically enhanced, we have performed a mosaic analysis of spaw activation in wild type and mutant clones. These studies, combined with functional analysis of downstream components, will further our understanding of the mechanisms by which asymmetric gene expression regulates cardiac morphogenesis.

218. Tbx1 interferes with Tgf-β signalling through the inhibitor Smad7 and impacts on great vessel development.

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DiGeorge syndrome is the most common microdeletion syndrome in humans, frequently caused by a 3Mb deletion of chromosome 22. The phenotype is characterized by craniofacial, cardiovascular, thymus, parathyroid and psychiatric defects. TBX1 localises within the commonly deleted region on 22q11 and is considered the major genetic determinant of the syndrome. Deletion of Tbx1 in animal models results in pharyngeal and cardiovascular defects, highlighting its role in the development of these structures. In an effort to study the pathways involved in these processes, we determined potential Tbx1 target genes through a microarray screen. Smad7 is an inhibitor of the Tgf-β pathway, and so far, several Tgf-β signalling genes have been described in heart development. We confirmed that Smad7 expression is diminished in Tbx1-/- mouse embryos and used a gene-trap mouse model to investigate the glandular, craniofacial and cardiovascular phenotype. We show an overlap between the Tbx1 null and Smad7 null phenotype as well as a genetic interaction between the two genes in great vessel development. Currently we are studying the mechanism involved in the morphogenesis of the symmetric embryonic arch arteries into the asymmetric mature vascular structure.

219. Genetic interaction of tbx1 with the Notch pathway effector her6 is required for pharyngeal development in the zebrafish

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Notch-pathway effector gene Hes1 is a putative down-stream target gene for Tbx1, which in turn is strongly implicated as a candidate gene for DiGeorge/Velocardiofacial Syndrome (DGS/VCFS). Using the zebrafish as a model, we show that tbx1 and her6 (zebrafish homologue of Hes1) are expressed in the pharyngeal tissues of zebrafish embryos. Furthermore, her6 expression is down-regulated in this region in both tbx1 mutant fish van gogh (vgo) and tbx1 morphants. Morpholino (MO)-based knock-down of her6 produces a similar phenotype to both the vgo mutant and tbx1 morphants, with embryos exhibiting comparable loss/abnormal patterning of caudal pharyngeal arch structures. Tg(sox10:gfp) fish exhibit abnormalities of neural crest migration with both morpholinos. Injecting sub-phenotypic doses of the tbx1 and her6 MOs in a simultaneous pair-wise fashion generates a synergistic increase in the observed pharyngeal defects. This implies a genetic interaction exists between her6 and tbx1, which was confirmed by rescue of the tbx1 morphant pharyngeal phenotype with injection of her6 mRNA. We are currently investigating whether this interaction between tbx1 and her6 is Notch-pathway mediated, or whether it is a further example of Notch-independent activation of her6.

220. A new locus for a syndromic form of Thoracic Aortic Aneurysms maps to chromosome 15q

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Background: Thoracic aortic aneurysms (TAA) are familial in at least 15-20% of the cases and can be classified in syndromic and non-syndromic forms. The TGF-beta signalling pathway plays a central role in the pathogenesis of both syndromic and non-syndromic TAA. Methods and results: We present a large four-generation family with a syndromic form of TAA. Thirty family members had an extensive physical and cardiologic examination. Eleven familymembers were considered affected on the basis of cardiac and/or skeletal and connective tissue abnormalities. Nine patients had an aortic or other large artery aneurysm. TAA patients had a high risk of aortic dissection or rupture at an early age. Many TAA patients had additional heart malformations, including mitral valve abnormalities, persistent ductus arteriosus and pulmonary valve stenosis. Histological examination of the aorta showed medial fragmentation, disarray of elastic fibres and excess of collagen deposition. After excluding mutations in the known syndromic TAA genes (FBN1, TGFBR1, TGFBR2, COL3A1), we performed a genome wide linkage analysis in this family using the Affymetrix 250K Nsp arrays. A new locus on chromosome 15q with a significant LOD score of 3.6 was identified within a critical region containing 120 genes. Sequencing of positional candidate genes is ongoing. Conclusions: The clinical phenotype overlaps with known TAA syndromes such as Marfan syndrome, Loeys-Dietz syndrome and vascular type Ehlers-Danlos syndrome. Our data provide evidence for a new locus for a syndromic form of TAA. Identification of this novel gene will provide insight into the pathogenesis of arterial aneurysms.

221. The ace-of-hearts mutant is defective in the establishment of cardiac left-right asymmetry

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In most vertebrate embryos, the heart is the first organ to display left-right asymmetry. During zebrafish embryonic development, asymmetric gene expression drives leftward displacement of the linear heart tube, and is followed by heart looping, which is crucial for proper alignment of the cardiac segments and subsequent heart function. To this day, our knowledge of the genes and mechanisms regulating the establishment of cardiac asymmetry remains limited. To uncover novel players and get insight into the molecular mechanisms controlling heart morphogenesis, we perform forward genetic screens for zebrafish mutants exhibiting heart asymmetry defects. Zebrafish offers significant genetic and embryological advantages, such as large progeny, fast development and straightforward microscopic observation. Additionally, the zebrafish two-chambered heart provides a simpler, though fully genetically relevant system for studying processes otherwise complex in many model organisms. We have identified ace-of-hearts, a mutant exquisitely defective for cardiac displacement and looping. Detailed analysis revealed that ace-of-hearts acts downstream of Nodal signaling and mutant embryos display no asymmetry defects in other organs. The mutation in ace-of-hearts was mapped to a premature stop codon on a gene encoding a protein convertase. Protein convertases activate proteolytically numerous proproteins amongst which BMP4, tgf-beta and nodal factors. However, their role in heart morphogenesis remains unclear. By assaying genetic interactions in mutants defective for predicted ace-of-hearts substrates and examining expression patterns of heart morphogenesis-specific factors in ace-of-hearts mutants amongst others, we are uncovering the place of ace-of-hearts in the pathways driving heart morphogenesis.

222. CRELD1, a genetic risk factor for congenital heart defects in humans, is essential for cardiovascular development.

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Previously our laboratory described the first genetic risk factor associated with atrioventricular septal defects(AVSD), called CRELD1. Recent work in our laboratory shows that CRELD1 may be working in concert with VEGF to regulate cardiovascular development. To determine the role of CRELD1 in development, we created a Creld1-knockout mouse. Characterization of the mouse demonstrates that Creld1 is required for proper cardiovascular development. Creld1 was found to be necessary for both normal development of the atrioventricular(AV) endocardial cushions, and for proper vascular modeling in both the embryo and yolk sack. We hypothesized that these cardiovascular abnormalities may be due to the misregulation of VEGF, an important factor in cardiovascular development. The ability of CRELD1 to regulate VEGF was supported by a human epidemiological study in which we found an association between AVSD cases, CRELD1 mutations and the VEGF-634C allele, a polymorphism associated with increased VEGF expression. This proposed CRELD1/VEGF interaction was further supported by employing a mouse endocardial cushion explant assay to determine if Creld1 controls the epithelial-to-mesenchymal transformation(EMT) required for valvuloseptal morphogenesis. We found that although EMT proceeds normally, the transformed cells respond differentially to VEGF when Creld1 is deficient. This indicates that Creld1 is a regulator of VEGF activity. The recent results from our human association studies in combination with data from our in-vivo and in-vitro mouse model studies support our hypothesis that CRELD1 controls cardiovascular development through modulation of VEGF activity. This novel CRELD1/VEGF interaction may explain the developmental origins of a subset of sporadic AVSD cases.

223. Increased Wnt signalling in early vertebrate development promotes atrial fate independently of head formation.

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Increased canonical Wnt signalling is suggested to produce a biphasic response in vertebrate cardiac development but also plays important roles in specifying the anterior embryonic structures. The effects on the morphology of the heart are poorly described. We evaluated the effect of increased Wnt signalling in vertebrate heart development using the zebrafish embryo and a mouse embryonic stem cell (ESC) assay of cardiogenesis. Although a biphasic response was seen for some genes in the ESC assay in response to the GSK3 inhibitor 6-bromoindirubin-3'-oxime (BIO), this was not seen for others and overall there was a reduction in numbers of embryoid bodies with spontaneous beating at all treatment stages. In zebrafish embryos, treated before completion of gastrulation, Wnt signalling produced the expected disturbances in body plan such that embryos either died or did not form normal cranial structures. In contrast, although embryos treated at the end of gastrulation appeared morphologically normal, there was a persisting reduction in total numbers of cardiomyocytes, combined with increased numbers of atrial cells. This correlated with a disturbance in the relationship between Nkx2.5 gene expression in the anterior lateral plate mesoderm and the expression of Ntl in the notochord. A corresponding increase in Mlc2a expression level was seen in the ESC assay. We have demonstrate that any positive effects of increased canonical Wnt signaling on production of cardiomyocytes are counter-balanced by severe disturbance of body plan. Moreover, even if cranial development is preserved ventricular hypoplasia and a shift towards increased atrial cardiomyocytes are still observed.

224. Time-regulation of cardiac chromatin remodeling complexes maintains a healthy heart

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In the heart, several stresses induce re-induction of fetal genes in association with cardiac hypertrophy and heart failure. However, no significant factors that regulate fetal genes expression via modulating chromatin structures have been identified. Here we show that NurD repressor-type chromatin remodeling complexes and their associated transcriptional repressor, Sall4 synergistically repress in progressive hypertrophic ventricles. In Sall4 mutant adult heart, fetal activator-type chromatin remodeling complexes, SWI/SNF-Baf was upregulated. To address whether abnormal expression of Smarcd3 directly caused heart failure, we generated two transgenic mice lines to overexpress Smarcd3 with α-MHC and CAG-CAT promoters. α-MHC-Smarcd3 TG mice caused abnormal hyper-trabeculare with ten times high number of cardiomyocytes in postnatal hearts exposed to hypertrophic stimuli. In these hearts, strong phospho-Histone H3–positive cells were observed with up-expression of several fetal genes, suggesting that stable Smarcd3 expression might block cardiomyocytes maturation. Furthermore, ECG analyses in these mice showed that the T-wave was completely down regulated. These results indicate that the new molecular pathway to cause cardiac hyperplasia relating with the chromatin remodeling factors in adult. We propose that Time- and Dose-dependent epigenetic signal pathway as chromatin/histone remodelers control healthy heart via regulating specific promoter regulation on genome in cardiomyocytes trabeculare and fetal gene program.

225. Mutation of the C-terminus of titin Leads to Hypertrophic Cardiomyopathy in Medaka Fish

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[Background] The Medaka fish represents an excellent model system for studying the cardiovascular system, since oxygen is delivered by diffusion and the embryo can tolerate the absence of blood flow. The aim of this study was to identify the gene responsible for hypertrophic cardiomyopathy. [Methods and Results] We used a chemical mutagenesis screen to identify the Medaka red blood island (rbi) mutant, which exhibited hypertrophic atrium and ventricle and lacked blood circulation, so that blood cells pooled in the blood island. Morphologically, the rbi heart developed normally. However, in the rbi embryo at 72 hours post-fertilization, the heart had lost elasticity and showed evidence of hypertrophic atrium and ventricle, and the heartbeat was weak from the first beat. These phenotypes were inherited as a recessive trait. Transmission electron microscopy of the rbi revealed reduced numbers of myofibrils and disrupted sarcomeric structures. Positional cloning revealed that rbi resulted from a 'causative' mutation in the titin gene. Titin, which is the largest known protein, binds myosin to the Z-disc in the sarcomere. We narrowed the critical region down to a 19-kb region of the C-terminus, which is important for myosin binding. RT-PCR analysis indicated higher expression levels of titin in the rbi. [Conclusion] Missense mutation of the C-terminal sequence of titin results in abnormal sarcomere assembly and hypertrophic cardiomyopathy in Medaka fish.

226. Comprehensive candidate approach for identification of sequence variants in cardiac transcription factors in patients with congenital heart defects

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Congenital heart defects (CHD) occur in nearly 1% of all live births and are the major cause of infant mortality and morbidity. Although improved understanding of genetic causes would provide insight into the pathobiological basis of CHD, the underlying genetic etiology of most CHD are still unknown. In order to explore genetic causes of CHD, we screened DNA derived from immortalized cell-line of 21 patients with non-syndromic persistent truncus arteriosus, and identified two GATA6 mutations. Both GATA6 mutant proteins lost transcriptional activity and failed to regulate downstream target genes associated with the outflow tract development. We have further screened DNA from immortalized cell-line of 516 patients with various non-syndromic CHD, and found nine sequence variants in four genes encoding cardiac transcription factors NKX2.5, MEF2C, GATA4 and GATA6 in eleven independent probands. These sequence variants were confirmed by analysis of patients' original genome and were not found in control population. Some of the mutant proteins showed significant decrease in transcriptional activity for the NPPA and other promoters for the cardiac development, and also decreased synergistic activity with other transcriptional factors. These findings suggest that the disturbance of regulatory circuit of these transcription factors may cause a subset of non-syndromic CHD. On the other hand, healthy family members of probands with some sequence variants of GATA4 and GATA6 had same nucleotide changes. It may be possible that secondary genetic and/or environmental factors may affect the penetration of CHD in these families. A comprehensive candidate approach for identification of sequence variants using immortalized cell-lines in combination with in vitro/vivo functional analyses would provide a way to clarify the pathogenesis of non-syndromic CHD that are considered to be multifactorial inheritance disorders.

227. Molecular patterning of the arterial trunk in early mouse embryos

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TBX1, encoding a T-box transcription factor, is the major DiGeorge (del22q11.2) syndrome candidate gene and is required for pharyngeal and cardiovascular development. Tbx1+/- mice present a high frequency of fourth arch artery anomalies and Tbx1-/- embryos have severe cardiac anomalies including a common arterial trunk and ventricular septal defect. DiGeorge syndrome patients present a high incidence of conotruncal defects including common trunk and tetralogy of Fallot. We have shown that the common arterial trunk in Tbx1-/- embryos has an aorta-like phenotype associated with severe reduction of a subpopulation of second heart field progenitor cells normally contributing to myocardium at the base of pulmonary trunk. Underdevelopment of subpulmonary myocardium is thought to be the primary defect in common human conotruncal defects including tetralogy of Fallot. Anomalous coronary artery patterning is observed in Tbx1-/- hearts: the left coronary artery courses ventrally, across a normally coronary-free region, to connect with an ostium overlying the right/ventral sinus of the arterial trunk. Semaphorin3c, encoding a neurovascular guidance molecule is expressed in a Tbx1-dependent domain in subpulmonary myocardium. Sema3c-/- embryos also display common arterial trunk although coronary artery patterning appears normal. Here we present a comparative analysis of the evolution of common trunk in these two models and investigate potential genetic interaction between these genes. Future subaortic and subpulmonary regions are prefigured in the E10.5 outflow tract. Using a candidate gene approach and microarray analysis at E10.5 we aim to identify additional genes expressed in subpulmonary myocardium that may contribute to conotruncal and coronary artery development.

228. Systematic survey of variants in TBX1 in non-syndromic Tetralogy of Fallot identifies a novel 57 base pair deletion that reduces transcriptional activity

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Tetralogy of Fallot (TOF) is common in individuals with hemizygous deletions of Chromosome 22q11.2 that remove the cardiac transcription factor TBX1. Here, we assess the contribution of common and rare genetic variants in TBX1 to TOF. We sought rare TBX1 variants by resequencing coding exons and splice-site boundaries. We investigated common TBX1 variants by genotyping 20 haplotype-tagging SNPs capturing all the common variation present at the locus. Association analysis was performed using the programme UNPHASED. TBX1 exons were sequenced in 93 non-syndromic TOF cases. SNP analysis was performed in 356 TOF cases, their parents, and healthy controls. Three novel variants not present in 1000 chromosomes from healthy ethnically-matched controls were identified. One of these variants, an in-frame 57 base-pair deletion in the third exon which removed nineteen evolutionarily conserved residues, decreased transcriptional activity by 37% in a dual luciferase assay ($p=0.005$). We have demonstrated that this mutation affected TBX1 protein stability. After correction for multiple comparisons, no significant associations between common genetic variants and TOF susceptibility were found. This study confirms that rare TBX1 variants with functional consequences are present in a small proportion of non-syndromic TOF but did not find association with common TBX1 variants.

229. The pharyngeal endoderm in cardiovascular patterning

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Cardiovascular development requires interactions between pharyngeal endoderm, ectoderm, endothelium and mesoderm-derived and neural crest-derived mesenchyme. Patients with del22q11 syndrome suffer from cardiovascular, thymus and parathyroid abnormalities and mutations in TBX1 have been implicated as an important genetic defect underlying the del22q11 phenotype. In Tbx1-null mouse embryos there is a reduction in the expression of the transcription factor Pax9 in the pharyngeal pouch endoderm at mid-embryogenesis. Mice lacking Pax9 are known to die perinatally with a cleft secondary palate and absent thymus, parathyroid glands, and ultimobranchial bodies. We now show that Pax9-null embryos have major cardiovascular defects including ventricular septal defect (VSD), double-outlet right ventricle (DORV), severely hypoplastic aorta, interrupted aortic arch (IAA), and aberrant right subclavian artery (A-RSA). The transcription factor Eya1 is thought to belong to the same genetic network as Pax9, and is expressed in the pharyngeal endoderm, mesenchyme and ectoderm in the mouse. Moreover, Eya1-null embryos die perinatally with abnormalities of pharyngeal pouch derivatives. We have found that embryos null for Eya1 also have cardiovascular defects including VSD, DORV, IAA and A-RSA. To investigate whether Pax9 and Eya1 genetically interact, we intercrossed Pax9+/-;Eya+/- mice and found that 90% of compound mutants died at around E10.5. Lack of X-Gal staining for Pax9lacZ in mutant embryos revealed loss of caudal pharyngeal pouch structures. In the mouse, Pax9 expression in the pharyngeal endoderm is vital for correct cardiovascular development. Our data suggest that Pax9 and Eya1, possibly together with Tbx1, genetically interact in the pharyngeal endoderm to control cardiovascular development.

230. The Role of Chap, A Novel Cardiac Z-Disk Protein, In Muscle Development And Function

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We recently identified Cytoskeletal Heart-enriched Actin-associated Protein (CHAP) as a novel cardiac-enriched gene in differentiating cardiomyocytes from human embryonic stem cells (ESC) (Beqqali et al., 2006). We identified two iso-forms; the longest isoform, CHAPa, contains a N-terminal PDZ-domain, a nuclear localization signal (NLS), and a C-terminal actin-association domain. The shorter isoform, CHAPb, lacks the N-terminal PDZ domain. CHAP is expressed in the Z-disc of striated muscle, associates with actin, binds to α-actinin and is essential for muscle and heart development in zebrafish (Beqqali, JCS in press). Here, we describe the CHAP knockout in mouse ESC (mESC), replacing the CHAP gene for LacZ (CHAP LacZ KI). These mESC were used to generate chimeric mice, which are currently bred for germline transmission. To investigate CHAP function in vitro, we differentiated the CHAP LacZ KI mESC into cardiomyocytes and analyzed their structure and function. Furthermore, we describe the isolation and sequence of the chick CHAP (GgCHAP) gene. The encoded GgCHAP protein is homologous to the mouse and human CHAPa isoform. In situ hybridizations for GgCHAP on chick embryos of different Hamburger and Hamilton (HH) stages showed expression of GgCHAP in heart (from cardiac crescent stage onwards), somites stages (HH15 onward), and in muscles of legs, wings (HH25 onward) eye and tongue (HH30). In future, intervention studies will be performed in chick. Supported by NHF grant 2006B218

231. George: A novel ENU-induced Tbx1 mouse mutation

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We have identified and characterized a novel ethylnitrosourea induced recessive mouse mutation (Geo) that has a phenotype resembling DiGeorge Syndrome (DGS). Phenotypic features include cardiac malformations (interrupted aortic arch, vascular ring, and ventricular septal defect), palatal cleft, inner ear defects, small or absent thymus and oedema. The mutation was mapped to a Chr 16 region containing Tbx1, a T-box containing gene that is a major candidate for DGS. Sequencing of Tbx1 revealed a non-synonymous point mutation at the end of exon3 that is predicted to change R160 to Q. Analysis of mRNA showed that the major effect of the mutation is skipping of exon 3, which is predicted to create an in-frame deletion of 34 residues from the Tbox. Geo does not complement a Tbx1 knockout confirming that it is a non-functional allele of Tbx1. As human and mouse Tbox sequences are identical, we sought to determine the functional significance of these 34 residues in the well-characterized human Tbx1c isoform. Our results show that this deletion, as opposed to the R160Q mutation, surprisingly does not affect the transactivation capacity of Tbx1 in reporter gene assays, suggesting that other mechanisms that affect function may be involved. To explore this we are investigating the effect of the loss of exon 3 on endogenous protein expression and the effect of this deletion on the Tbx1 protein interaction network.

232. Noninvasive, High-Resolution Ultrasound Imaging To Evaluate Cardiac Function In Teratogen-Exposed Rat Foetuses.

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Structural defects are readily identified at parturition in native or fixed tissues; however, their function consequences are harder to ascertain. Our goal was to assess the utility of the Vevo660 high-resolution ultrasound biomicroscope (VisualSonics, Toronto, ON) for evaluating cardiac defects in Sprague-Dawley rat embryos/foetuses exposed to dimethadione, a known cardiac teratogen. Doppler, m-mode and b-mode images were collected from foetuses ranging from gestational day (GD) 14 to post-natal day (PND) 1, both in utero and following externalization of the rat uterus. Foetal exposure to dimethadione was associated with quantitative changes in morphological and functional (haemodynamic) parameters indicative of improper heart development, and consistent with post-mortem histology. The ability to monitor cardiac anomalies longitudinally was limited by an inability to control in situ embryo/foetal orientation and to accurately identify a given embryo within a litter over multiple days. Although externalization of the uterus enabled greater control over image orientation, the difficulty in controlling physiological parameters, combined with its invasiveness, made longitudinal studies non-feasible for our purposes. Despite these limitations, imaging in all three modes provided quantitative measures of parameters that were previously only assessed qualitatively. These included ventricular septal defects, persistent truncus arteriosis (PTA), thinning of ventricular walls and ventricular hyperplasia. In addition, high frequency ultrasound permitted the monitoring of functional (haemodynamic) changes not typically measured in post-mortem developmental toxicity safety studies. We conclude that high frequency ultrasound is an excellent tool to assess cardiac structure and function following in utero exposure to teratogens. (Funded by Pfizer Global Research and Development)

233. Genetic modifiers predisposing to congenital heart disease in a sensitized population

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Congenital heart disease (CHD), the most frequent birth defect in human beings, has long been associated with complex genetic syndromes. Trisomy for human chromosome 21 (Hsa21) is the most frequent risk factor for CHD. The frequency of CHD in individuals with Down syndrome (DS) is about 50 times higher than in the general population. However, 50% of people with DS have a normal heart, thus, trisomy 21 is not sufficient to cause CHD. Additional genetic components may contribute to the perturbations resulting from gene dosage effects in trisomy. We have identified polymorphisms in the CRELD1 gene that are associated with CHD in individuals with DS. To establish a biological basis for this interaction, we have initiated studies of CHD in animal models of Down syndrome. The Creld1 null allele was introduced into Ts65Dn mice, which are trisomic for orthologs of about half of the genes on Hsa21. We provide direct evidence that reduced Creld1 expression exacerbates septal defects in Ts65Dn, Creld1+/- trisomic mice. A genome scan has been undertaken to find more disomic CHD modifiers by comparing the genetic backgrounds of the Ts65Dn, Creld1+/- pups with or without septal defects. To focus the search for trisomic genes that interact with Creld1, we introduced the Creld1 null allele onto Ts1Cje mice. These mice are trisomic for about 80% of the Mmu16 genes that are over-represented in Ts65Dn. Surprisingly, no exacerbation of septal defects was detected in Ts1Cje, Creld1+/- mice. This suggests that one or more of the 37 conserved genes that is trisomic in Ts65Dn but disomic in Ts1Cje is necessary for the Creld1 interaction. Evidence regarding these candidate genes will be presented.

234. Deconstructing the role of Pcsk5 in cardiovascular development.

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In a phenotype-driven recessive screen of ENU-mutagenised mice, we have identified a mutant (Vcc) with a pleiotropic phenotype with cardiac, tracheo-esophageal, anorectal, axial skeletal antero-posterior patterning defects, limb malformations, presacral mass, renal and palatal agenesis, and pulmonary hypoplasia. Multiple cardiac anomalies included dextrocardia, ASD, VSD, CAT, vascular ring, right-aortic arch and hypoplastic arterial duct. The phenotype showed a recessive inheritance pattern with 100% penetrance and appeared to result from a C470R mutation in the proprotein convertase PCSK5 (PC5/6, SPC6) [Szumska et al. Genes&Dev. 2009]. To explore further the cell-autonomous role of PCSK5 in cardiac development, we have employed a conditional knockout approach (in collaboration with Annik Prat & Nabil Seidah, who generated the Pcsk5 floxed allele). The heart, although induced by endodermal signals, is essentially mesodermal in origin, with a minor contribution from the ectodermal neural crest. These embryonic lineages arise from the epiblast. We have deleted Pcsk5 in the mesoderm using Mesp1Cre and Nkx2-5Cre, in neural crest using Wnt1Cre and in the pharyngeal arches using Hoxa3Cre. We also used Sox2Cre to delete Pcsk5 at an early time-point in all three embryonic germ layers (epiblast KO). We studied Pcsk5null/flox;Cre+ embryos in comparison to their Pcsk5wt/flox;Cre+ littermates. Epiblast deletion of Pcsk5 generated full mutant phenotype observed both in Vcc mutant and in Pcsk5KO. We have observed heart anomalies (VSD, ASD, DORV, TGA) in Pcsk5null/flox;Mesp1Cre+ and not in their control littermates. However, there were no obvious structural cardiac defects, detectable by MRI, in embryos with Pcsk5 deleted in neural crest and pharyngeal arches.

235. Mutations in the sarcomere protein gene MYH7 in Ebstein`s anomaly

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Background- Ebsteins`s anomaly is a rare congenital heart malformation characterized by adherence of the septal and posterior leaflets of the tricuspid valve to the underlying myocardium. The genetic basis of this disorder is largely unresolved. Associated abnormalities of left ventricular morphology and function have been observed. We speculated that mutations in the sarcomere protein gene β-myosin heavy chain (MYH7) known to cause familial cardiomyopathy - hypertrophic cardiomyopathy (HCM) and left ventricular noncompaction (LVNC) - may be associated with Ebstein`s anomaly. Methods and Results- Mutational analysis in a cohort of 131 unrelated probands with Ebstein´s anomaly was performed by next generation sequencing (Roche Genome Sequencer FLX system) and direct DNA sequencing of MYH7. One half of the patients had no associated cardiac anomalies, the most common associated cardiac malformation being atrial septal defect (ostium secundum type, ASDII) and 6 patients had left-sided cardiomyopathy. Heterozygous mutations were identified in 8 of 131 samples (6%). 7 distinct mutations were found of which 5 were novel and 2 were known to cause HCM. All mutations except for one 3-bp deletion were missense mutations. In 5/8 probands with MYH7 mutations LVNC was identified additionally to Ebstein´s anomaly. In 123 probands without MYH7 mutations there was only one patient with additional HCM. MYH7 mutations segregated with LVNC in 3 autosomal dominant kindreds and familial Ebstein´s anomaly was found in one kindred. Conclusions- Ebstein`s anomaly is within the diverse spectrum of cardiac morphologies associated with mutations in the gene encoding β-myosin heavy chain. Our findings implicate that left ventricular noncompaction is not uncommon in patients with Ebstein´s anomaly and MYH7 mutations.

236. Molecular and functional characterization of Pitx2 myocardial ko mice

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Understanding the molecular pathways that regulate cardiac growth and function is crucial to prevent and cure heart disease. The homeobox gene Pitx2 plays an important role in these processes, since it is required to confer left atrial identity and to regulate asymmetric ventricular remodelling. We have generated a myocardial specific Pitx2 ko by crossing Pitx2 floxed and cardiac Troponin T (cTnT) Cre mice, which results in loss of the gene from the very early phases of cardiomyogenesis. The cTnT Cre-Pitx2 ko (cTP komyo) is incompatible with life and recapitulates almost completely the cardiac phenotype of the constitutive Pitx2 null mice. We have found that myocardial deletion of the Pitx2 gene lead to the presence of bilateral SAN, suggesting that the presence of the gene in the left myocardial precursor cells plays a repressive role in the genetic pathway determining SAN formation. Early genetic targets of Pitx2 are currently being investigated. Moreover, Pitx2 myocardial mutant hearts present impaired maturation of the conduction system. Functional characterization of the mutant embryonic and adult hearts by optical mapping and ECG analysis is currently being performed. Comparison with the constitutive Pitx2 ko model will be presented.

237. Genome-wide screen of non-syndromic Tetralogy of Fallot patients identifies a duplication in Connexin40

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Objective: To assess the contribution of copy number variation (CNVs) to non-syndromic Tetralogy of Fallot (TOF) Methods: We screened 206 TOF probands who did not have a 22q11.2 deletion and their unaffected parents on the Affimetrix Genome-Wide Human SNP 6.0 Array and analysed the data using the Birdseye algorithm. Results were confirmed by two other independent platforms: Illumina 660W-Quad and custom Multiplex Ligation-dependent Probe Amplification (MLPA) assay. Results: Among all the copy number variation observed, we identified two CNVs in chromosome 1q21.1. Both were duplications; one de novo 0.8Mb duplication spans several genes, while the smallest duplication contains only GJA5/Connexin40 (CX40), a gap-junction protein expressed in the heart. Conclusion: Chromosome 1q21.1 deletions have been previously described in TOF patients (Greenway et al, 2009). This case narrows the region on 1q21.1 to a single gene, Connexin40. Conotruncal malformations, including TOF, occur in the Cx40 mouse mutant (Gu et al, 2003). We conclude that copy number variation of Connexin40 is likewise associated with Tetralogy of Fallot in humans.

238. The great intrathoracal arteries of early wild type OF1, Parkes, and C57Bl/6 mouse fetus

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Analysing the morphological phenotype of mice carrying targeted or random gene mutations is an useful approach for researching hereditary cardiovascular diseases. Homozygote offspring of such mutants, often die in utero enforcing morphologic analysis of embryos. Our presentation aims at presenting objective topological and metric descriptions of the great intrathoracal arteries of early mouse fetus (developmental stage 23 according to Theiler (TS23)) of the Him:OF1 (n=60), NIMR:Parkes (n=30), and C57Bl/6 (n=30) strains. Employing the "High Resolution Episcopic Microscopy" (HREM) technique, we generated digital volume data sets (true spatial resolution of 2x2x2 µm3/voxel) and three-dimensional (3D) surface models of the great intrathoracal arteries. The 3D models were used: Firstly for topological analysis. Secondly for defining reproducible positions along the blood vessel trees. Thirdly for defining virtual section planes, which, in all spatial directions, cut through the original volume data exactly perpendicular to the longitudinal axis of the blood vessel segment to be measured. In the virtual sections the blood vessel perimeter was measured and the diameter was calculated from the perimeter. We present topologic descriptions of the great intrathoracal arteries, and measurements and relations of the lumen diameters of the ascending aorta, pulmonary trunk, ductus arteriosus, and descending aorta distal to its communication with the ductus arteriousus and proximal to the origin of the 1st segment artery. Finally we provide comparisons between the different strains and discuss the significance of our data for diagnosing vascular malformations in mutant mouse embryos.

239. Evaluating a novel procedure for measuring the diameters of the great intrathoracal arteries of mouse embryos

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Careful topologic and numeric analysis of the morphological phenotype of mutant mouse embryos is the basis for researching gene function and the genesis of cardiovascular malformations. Objective diagnosis of the stenosis or dilation of embryonic blood vessels is a major challenge. In this presentation we evaluate a recently published procedure for measuring the dimensions of the great intrathoracal arteries of mouse fetus. By employing the High Resolution Episcopic Microscopy (HREM) technique we generated volume data (voxel size: 1.07x1.07x2 µm3) of 60 mouse embryos of the Him:OF1 strain of early to late developmental stage 23 according to Theiler. Each of two independently working diploma students randomly received 30 of these data sets. The students created 3D surface models of the great intrathoracal arteries and used them for defining comparable measurement positions (aorta ascendens, pulmonary trunk, and ductus arteriosus in their middle; aorta descendens distal to the ductus arteriosus and proximal to the 1st segmental artery). Then they defined virtual planes cutting through the original volume data in all spatial directions perpendicular to the longitudinal axis of those blood vessel segments. In these planes they measured the perimeters of the blood vessel lumen. Comparisons with t-test revealed no significant differences between the values measured by each student. We consider this as proof, that the employed measurement procedure is capable of producing accurate, highly objective, reproducible results. Furthermore the measurements themselves represent meaningful reference data for identifying stenosis or dilation of the great intrathoracal arteries of early OF1 mouse fetus.

240. Role of Dhand in A Familial Case of Congenital Heart Disease

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Members of the basic helix-loop-helix (bHLH) transcription factor family are known to regulate the specification and differentiation of many cell lineages during embryogenesis. The bHLH proteins dHAND and eHAND are expressed in the cardiac tissues, craniofacial region and limbs in a complimentary and overlapping fashion during embryonic development. Studies using gene knockout have shown that dHAND and eHAND null mice embryos die early due to defects in the development of the right ventricle and placenta, respectively. As cardiac abnormalities occur with an incidence of one per 100 live births and represent the highest percentage of all congenital malformations accounting for about 30%, we decided to investigate the role of dHAND in a familial case of congenital heart disease.
In this study, we report a heterozygous mutation G205V in the gene encoding the dHAND that has been found in 7 out of the 17 members of the family and in none of the 44 patients with different phenotype, nor in the 36 healthy individuals included in this study. Therefore, after identifying a novel missense heterozygous mutation, we conducted site directed mutagenesis to study the effect of the amino acid substitution. The mutation in the C terminal region of dHAND slightly reduced its binding affinity to DNA without affecting its cellular localization. Finally, we identified Tbx5 and Tbx20 as new partners for dHAND and found that the mutation affects the interaction of dHAND with Tbx5 but not with Tbx20.

241. Endocardial origin of BAVs in Gata-5 null mice

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Congenital heart defects in humans occur in 1% of live birth. The majority involve the valves and septa, which originate from endocardial cells. Bicuspid aortic valve is the most common congenital cardiac malformation in humans and occurs in 1-2% of the population. Although it's clear that BAV is a heritable trait, few genes have been linked to this defect. In human, only one gene, Notch1 has been directly associated with BAV. In mice, inactivation of eNOS lead to low penetrance of BAVs. Thus, the genes and mechanisms that lead to BAV formation remain poorly understood. Over 26% of Gata5 null mice have bicuspid aortic valves (BAVs). The distal outflow tract cushion is made of different sources of mesenchyme, including neural crest mesenchyme, mesenchyme from pharyngeal area and mesenchyme of endocardial origin. In order to define the cell type that causes BAVs in Gata5 null mice, we generated mice with specific deletion of Gata5 in endothelial cells, using the Tie2-cre transgenic mice. Here, we show that a subset (21%) of Tie2-cre;Gata5Flox/Flox mice have bicuspid aortic valves, with a ratio that is practically identical to that of Gata5-/- mice. This result suggests that the presence of BAVs in Gata-5 null mice is of endocardial origin and point to an essential role of Gata5 in endocardial development and valve formation.

242. SMAD6 Mutations in Patients with Congenital Cardiovascular Malformation

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Bone morphogenetic proteins (BMPs) regulate many processes during development, including cardiac development. SMAD6 functions as an inhibitory SMAD which preferentially inhibits BMP signalling. The SMAD6 knockout mouse is characterised by cardiac valve and outflow tract defects, including aortic ossification. We hypothesised that rare functional variation in SMAD6 could predispose to congenital cardiovascular malformation (CVM). Methods: We sequenced coding exons of SMAD6 in 459 CVM patients of white Europeans. To test for functional effects, wild-type and variant SMAD6 proteins were expressed in C2C12 cells and their capacity to inhibit ALK3 activated expression of a BMP-responsive reporter, or to inhibit osteogenic differentiation (using an alkaline phosphatase assay) was assessed. Results: We identified two novel non-synonymous variants, P415L and C484F, that were not present in 1000 ethnically-matched controls. P415L was identified in a patient with congenital aortic stenosis and C484F was identified in a patient with coarctation and calcification of the aorta. Both mutations are in evolutionarily conserved amino acid residues and are predicted to be damaging by in silico analysis. This was confirmed in functional assays as both SMAD6 variants failed to inhibit BMP signalling compared with wild-type SMAD6. The P415L mutant appeared to be hypomorphic whereas C484F appeared to be a null allele. Conclusions: This is the first time that functional mutations in SMAD6 have been described in patients with CVM, specifically those with calcific aortic malformations. Our data suggest that dysregulation of the BMP signalling pathway may be an important factor in CVM.

243. PITX2 is highly expressed in the adult left atrium and a mouse model with reduced expression displays inducible atrial fibrillation and action potential shortening

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Intergenic variations on chromosome 4q25, close to the PITX2 locus, are associated with atrial fibrillation (AF). PITX2 encodes a transcription factor, expressed as several protein isoforms, with roles in the development of L-R asymmetry, pulmonary vein myocardium and sinuatrial node. Using isoform-specific RT-qPCR, we find the left atrium (LA) of adult human and mouse hearts express PITX2 at levels comparable to skeletal muscle (highest known adult expression),whilst the other chambers are >100 fold lower. PITX2c isoform predominated, with levels >100 more than a or b, in both species. Levels in atria from patients with sustained AF were not different to patients in sinus rhythm. Mice heterozygous for Pitx2c showed LA Pitx2c expression of ~60% wild-type (WT). Echocardiography and histology in Pitx2c+/- showed no overt abnormality in chamber size, morphology or function, except for slightly elevated RV pressure. Spontaneous AF did not occur more often in Pitx2c+/-, but isolated beating Pitx2c+/- hearts were susceptible to AF during programmed stimulation. At short paced cycle lengths, atrial action potential durations (APD) were shorter in Pitx2c+/-. The ß-receptor agonist orciprenaline abolished inducibility of AF and reduced the difference in APD. Pathway and FatiGO analyses of expression arrays, comparing Pitx2c+/- to WT, LA & RA separately, revealed clusters of genes related to gap and tight junction regulation, melanogenesis, and calcium ion binding affected by Pitx2. These findings demonstrate a physiological role for PITX2 in the adult heart, support the hypothesis that dysregulation of PITX2 causes susceptibility to AF, and provides clues to potential molecular mechanisms.

244. Transposon traps the heart.

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With the aim to maximize the experimental utility of zebrafish as a model for cardiovascular studies, we generated about twenty cardiac enhancer trap (CET) transgenic zebrafish lines using transposon-mediated enhancer trap. One subset of CETs defines cell layers of the heart - endocardium, myocardium and epicardium, whereas another CET subset defines various structural elements of the heart, including its chambers – the atrium, ventricle as well as the valve at the atrio-ventricular boundary and bulbus arteriosus. Most of these expression domains are maintained into adulthood. Furthermore, genomic information delineating the transposon insertion sites provided clues about genes which expression was tagged by the insertions resulting in identification of genes that may function in the respective cell-types or regions of the heart. The expression pattern of EGFP in some CETs is unique and recapitulates expression of genes flanking the transposon insertion site. The CETs enabled us to capture the dynamics of the embryonic heart beating in vivo using fast scanning confocal microscopy coupled with image reconstruction, producing three-dimensional movies in time (4D) illustrating region-specific features of heart contraction. In addition, some CETs provide a unique opportunity to carry out functional analysis on the poorly understood cardiac conduction system (CCS), which is pivotal to the initiation, maintenance and coordination of the rhythmic contraction of the heart. This collection of CET lines represents a toolbox of markers for in vivo studies of heart development, physiology and disease.

245. Genetic modifiers of intracardiac defects in humans with velo-cardio-facial /22q11.2 deletion syndrome

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Approximately 70% of 22q11.2 deletion syndrome (22q11DS; velo-cardio-facial/DiGeorge) patients have intracardiac and/or aortic arch malformations. We have performed a genome-wide association study on subjects with the 22q11.2 deletion to find genetic risk factors for intracardiac malformations using Affymetrix 6.0 microarrays. The arrays contain 1.8 million genetic variations (SNPs, CNVs). In the first set, we analyzed data from 169 subjects with tetralogy of Fallot (TOF) and 190 with normal intracardiac anatomy as determined by echocardiography. We found no association to SNPs on the remaining allele of chromosome 22q11.2, ruling out common variations as a significant contribution. We sequenced TBX1, a major gene for the syndrome, on the other allele, in 376, 22q11DS subjects but found no mutations. Several known candidate genes were ruled out by this approach. We reasoned that genetic risk factors might lie elsewhere in the genome. One locus on chromosome 12p12 showed significant association, p= 9x10-8. Other loci (4q12-13, 2p21) were just below significance. Besides TOF, other intracardiac defects (VSD, ASD, PTA) and/or aortic arch malformations (IAA-B, PS) occur in 22q11DS patients. All are being reanalyzed with a larger dataset from 700 Affymetrix chips on 22q11DS patient DNAs, as qualitative as well as quantitative traits. Large, rare copy number variations will be validated by FISH mapping. In addition to studies of phenotype, we performed an association study to identify risk factors for generating the deletion and found two loci near genome-wide significance (5q23, 21q22) that will be reanalyzed with the new dataset. Supported by NIH, HL84410.

246. Closure of the aortopulmonary foramen in the developing heart (no poster)

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Theodore Kramer, writing more than 50 years ago, commented that partitioning of the distal outflow tract was so well-known as to preclude the need for further discussion. Despite his optimism, the topic remains controversial. With this in mind, we have reviewed development of the outflow tract in man and mouse, using three-dimensional reconstructions, along with scanning electron microscopy, to elucidate the formation of the intrapericardial components of the aorta and pulmonary trunk. The key step is closure of an aortopulmonary foramen, bounded proximally by the distal ends of the spiralling outflow cushions, and distally by the dorsal wall of the aortic sac between the origins of the arteries of the fourth and sixth pharyngeal arches. As noted by Kramer, this dorsal wall represents the aortopulmonary septum. Closure of the foramen between the wall of the aortic sac and the distal ends of the cushions, themselves fusing to divide the distal outflow tract, places the developing aortic channel in continuity with the fourth arch, and subsequent to obliteration of the right sixth arch, the pulmonary channel in communication with the developing pulmonary arteries and the arterial duct. Cells from the neural crest invade the cushions prior to their fusion, while additional cells from the second heart field invade the parietal walls of the outflow tract. Fusion of the dorsal wall of the aortic sac with the cushions, however, is necessary to produce the adjacent walls of the aorta and pulmonary trunk. Failure of fusion explains well the occurrence of aortopulmonary windows.

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