Newcastle Cardiovascular Development meeting 2016

European Society of Cardiology Working Group for Development, Anatomy and Pathology

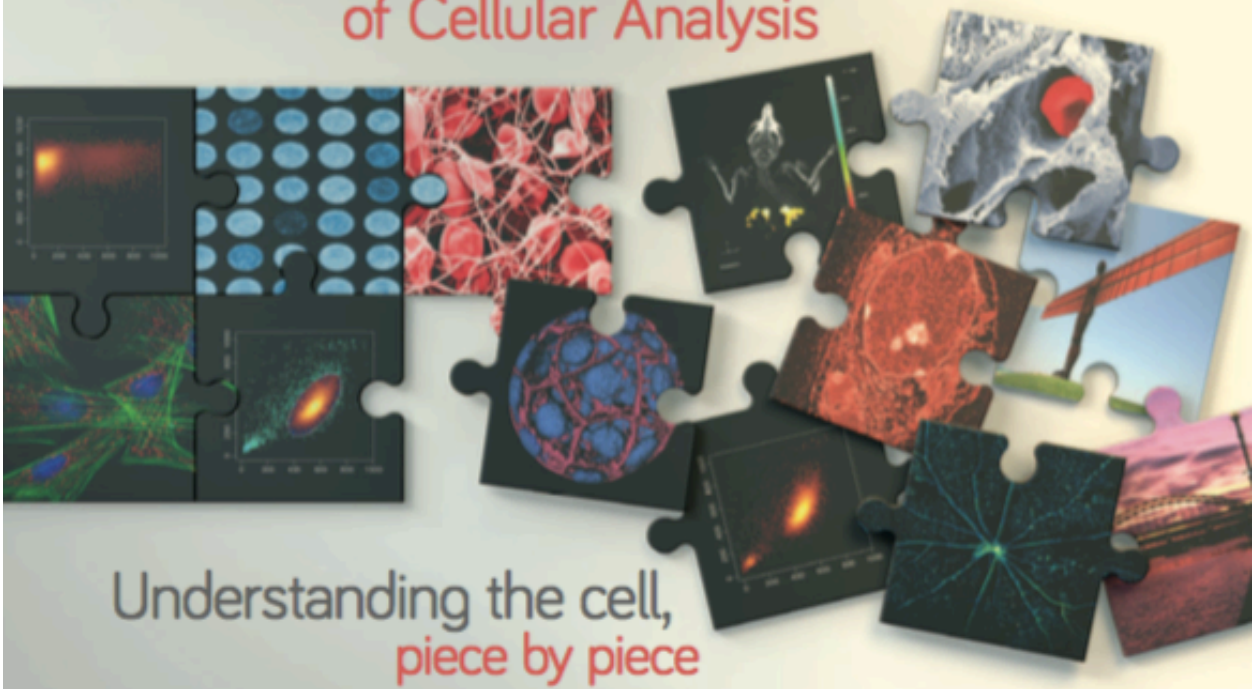
November 15-17th, 2016

Scientific Facilities Conference
2017 | Tuesday 28th March
Newcastle University, David Shaw Lecture Theatre
Medical School, Framlington Place

Faculty of Medical Sciences
Scientific Facilities

Newcastle University

Practicalities
of Cellular Analysis



Understanding the cell, piece by piece

Light Microscopy | Electron Microscopy | Flow Cytometry | Infectious Diseases | Pre-clinical *in vivo* imaging

Speakers

Hervé Luche
Centre d'Immunophénomique

Martin Picard
Columbia University

Mike Carroll
Newcastle University

Michael Kelly
Leicester University

Jack Lesley
Newcastle University

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Event

Registration

Delegates: FREE
Commercial representation: £300 + VAT per stand
See website to register

Poster Presentation Session

Abstract submissions by 24/02/2017

See website for further details

Enquiries

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Welcome

On behalf of the European Society of Cardiology Working Group - Development, Anatomy and Pathology and the Newcastle University Cardiovascular Research Centre we are pleased to welcome you to the annual Cardiovascular Development meeting held this year for the first time in Newcastle upon Tyne. We are delighted to have participants coming from as far afield as Israel, the Lebanon, USA, Brazil and Japan, as well as nine European countries.

The goal of our meeting has always been to provide a highly interactive forum for researchers in the field of cardiovascular development, anatomy and pathology with a European flavour. The meeting is designed to be large enough to provide a wealth of thought provoking topic areas, but small enough to allow participants to meet and get to know each other. Our hope is that this annual meeting and our working group will rise above the uncertainties and changes within Europe and, in addition to reinforcing our existing collaborations and friendships, will also create new ones.

Some of you will have visited Newcastle before and already experienced this friendly and accessible University city. Everywhere is within easy walking distance. We will provide you with up to date information on restaurants and bars and hope you will take the opportunity to sample the city at night. If you have questions or problems, please do not hesitate to speak to the local organising team or the ESC conference team.

As always we are very grateful to the European Society of Cardiology for supporting our meetings and recommend that you join our working group of the ESC - it is free and provides access to reduced fees to our meetings. We would also like to thank Newcastle University who have generously sponsored the meeting in recognition of the newly opened Newcastle University Cardiovascular Research Centre (www.ncl.ac.uk/cardio). Finally, we would like to thank our industry sponsors: Biorad, IonOptix, NEB, VWR, Nikon and Acea Biosciences for supporting this meeting. Please take time to meet them at their stands at the Great North Museum during Wednesday.

This year we will be holding a competition to win a copy of the ESC development text book, which is in the process of being printed by Oxford University Press. This book is the definitive textbook of cardiac development. To enter the draw you will need to have your entry card marked by each of the sponsors and give them to the ESC organising team by the end of Wednesday 16th November. The Draw will be made at the end of the final session on Thursday 17th November. The winner will receive their copy as soon as it becomes available.

Finally, please remember to fill in your feedback form, which will help us to improve our future meetings.

Deb Henderson
Bill Chaudhry

Venues

Tuesday November 15th

The meeting will be held in the IGM Lecture Theatre, Biomedicine West, Institute of Genetic Medicine, Newcastle University, International Centre for Life. In the evening we will walk across the square to the Life Exhibition Centre for the conference reception.

Wednesday 16th –Thursday 17th November

The meeting will be held in the conference suite on the first floor of the Great North (Hancock) Museum. You will be able to look around the exhibits and, in particular, we recommend you view the exhibits from Hadrian's Wall.

WIFI

There is WIFI available in the IGM and also within the Hancock using the University WIFI network. We will provide you with a personal wifi code when you register to get you on-line.

Speakers

The speakers have been asked to up-load their presentations before the first session of the morning or during lunch to ensure we have smooth transitions between speakers. Both PC and Mac formats will be available. We will be absolutely strict on timing and will be using a visual timer. Green light during talk time, amber will warn of the final minute and red is an absolute cut off!

Posters

We will look at odd poster numbers on Wednesday and even number posters on Thursday lunchtimes - but please display your poster throughout both days. Be sure to be at your poster for when the markers arrive. There are prizes...

Prizes

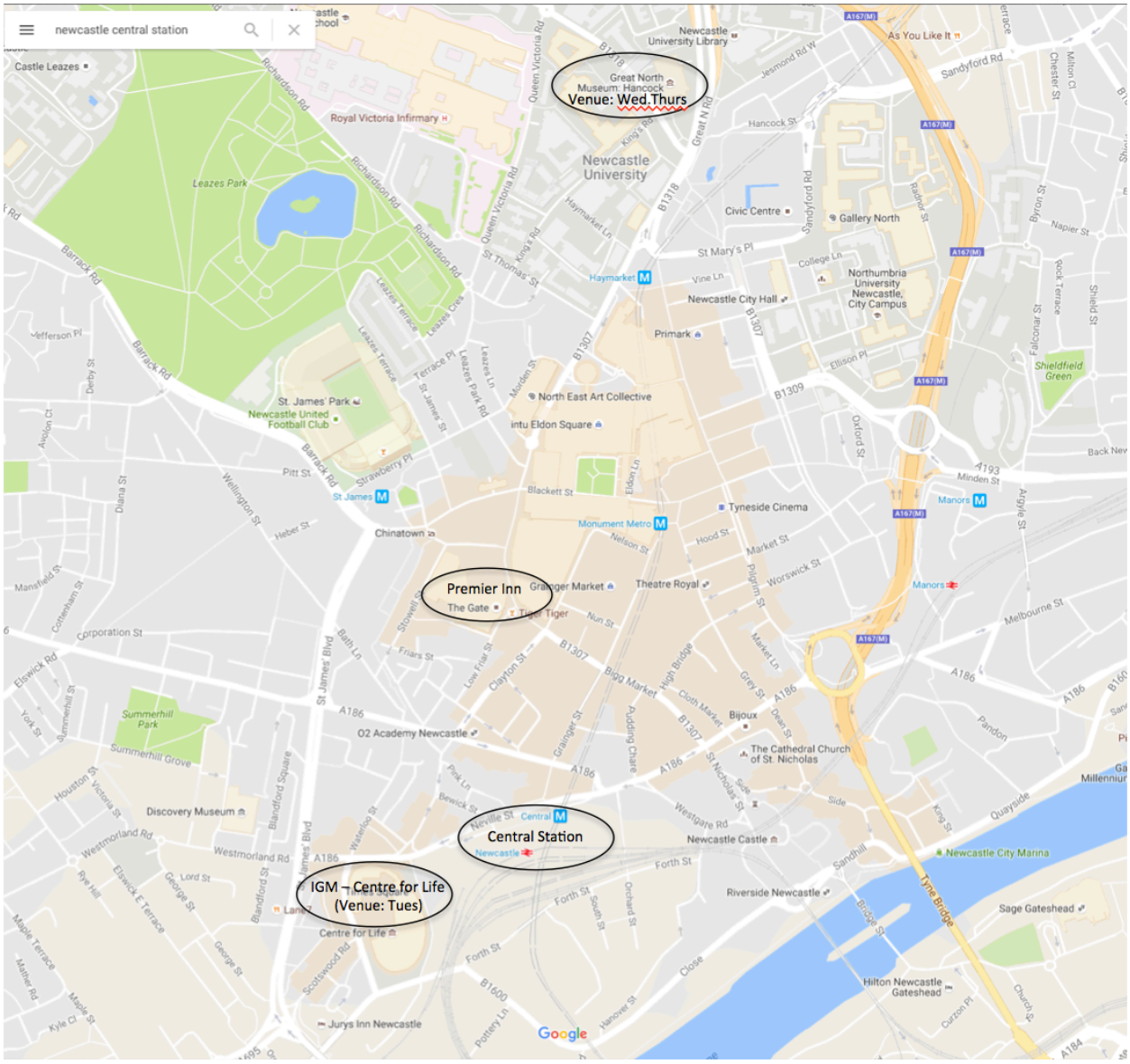
We will be awarding the Pexieder prize for the best podium presentation and additional prizes for best oral and poster presentations. These will be announced after the final session.

Remember our meeting and the sponsors

Take care not to drop the conference mug. You will need it to drink English tea when you get home.

How to get to the meeting when you arrive in Newcastle

From Newcastle International Airport to Institute of Genetic Medicine (Centre for Life)



1. Metro train from Airport to Newcastle Central Station (approx. 40 min). Follow signs for metro/taxi and you will easily find the metro platform at the exit of the airport building. There are machines to buy tickets that take UK coins but also credit cards. There is only one platform with two tracks - get on any train that is waiting as it comes to the correct place. If you are coming directly to the conference get off at Central. Exit through the Railway station and you will see the Centre for life as a large blue building about 100m on the left. If you are going to the Premier Inn Hotel you may wish to get off at Monument. Walk up the road that has lots of buses on, past a war memorial (Old Eldon Square) in the direction of the Gate. If you need help ask anyone where the old CO-OP building is!
2. Taxi to Centre for Life or Premier Inn (approx. 25 min). Cost approx. £20. The airport taxi company is the only one allowed to pick up at the airport and is professional and courteous. Tips are not essential but we usually round up to a full pound.

From Newcastle Central Station to Premier Inn

1. Exit through the Railway station and you will see the Centre for life as a large blue building about 100m on the left. You can get a taxi immediately outside, but it's only about 7 minutes walk to the Premier Inn. Get there by crossing the road by the Centre for life and walk up Clayton Street, past the Cathedral Cafe. At the next crossroads go straight on past "Richer sounds" electrical store. Turn left left at the next crossroads (on to Newgate Street). The hotel is on your left, just past The Gate cinema complex.

To the Hancock from Premier Inn

Walk left on exiting the Premier Inn and straight up Percy Street. Carry on past the university buildings until you reach Quilian Brothers teahouse on the corner. Turn left immediately after the teahouse and walk up Claremont Road for approximately 30m and use the crossing. Enter by the “Schools and Groups” entrance.

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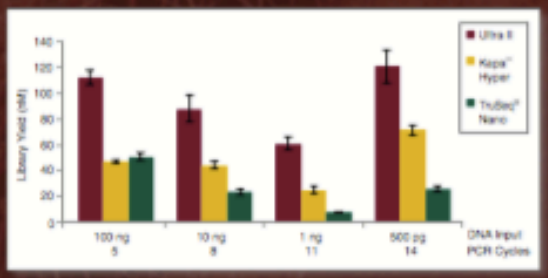

Libraries were prepared from Human NA19240 genomic DNA using the input amounts and numbers of PCR cycles shown. Manufacturers' recommended protocols were followed, with the exception that size selection was omitted.

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Scientific Programme

Tuesday November 15th: IGM Lecture Theatre, Centre for Life

12.00	Registration and lunch	
13.15	Deborah Henderson	Welcome
Session 1:	Chairs: Lucile Houyel and Robert Anderson	**Development, Anatomy and Pathology of the cardiac outflow tracts**
13.20	Robert Kelly	Outflow tract development: the regulation of second heart field progenitor cell deployment in the mouse
13.50	Federico Tessadori	Cardiac looping is driven by heart tube-intrinsic morphogenesis in zebrafish
14.05	Jörg Männer	Cardiac looping may improve the efficiency of valveless pumping in the embryonic heart tube.
14.20	Patricia Garcia-Canadilla	Detailed assessment of cardiac anatomy and microstructure in congenital heart disease by X-ray phase-contrast synchrotron radiation-based micro-CT
14.35	Robert Anderson	Development and maldevelopment of the ventricular outflow tract
15.05	Coffee break	
15.45	Vi-Hue Tran	Morphology of ventricular outflow tract malformations
16.15	Jill Hikspoors	Development of the (supra-) hepatic portion of the inferior caval vein
16.30	Bill Chaudhry	Morphological analysis suggests alternative classification of HLHS
16.45	Anthony Firulli	Testing a Somatic Model of Hypoplastic Left Heart Syndrome
17.00	Asif Hassan	Surgical repair of outflow tract malformations
17.45-20.00	Reception at LIFE exhibition centre, drinks and canapés Including: Gunther von Hagens' "Animals Inside Out"	

Wednesday 16th November: Great North Museum (Hancock)

8.30	Registration	Posters and speaker set up

Session 2	Chairs: Antonio Baldini and Pete Scambler	**Cardiac progenitors and cardiogenesis**
9.00	Antonio Baldini	Cardiac progenitors transcription program and heart defects
9.30	Sonia Stefanovic	Retinoic acid-dependent regulation of cardiac genes
9.45	Simon Bamforth	*Tbx1* genetically interacts with *Pax9* to control fourth arch artery morphogenesis
10.00	Srinivasan Sakthivel	Whole exome sequencing identifies a novel candidate gene for congenital heart disease
10.15	J.A. Guadix[1]	Differentiation of hESC cells into the cardiac 'venous pole'/'cardiac inflow' cell types

10.30	Coffee, trade stands and poster viewing

Session 3	Chairs: Shane Herbert and Jose-Luis de la Pompa	**Endothelium, endocardium and flow**
11.00	Shane Herbert	Coordination of Collective Cell Migration in Angiogenesis
11.30	Nicoletta Bobola	Transcriptional control in the developing heart circulation
11.45	Salim Abdelilah-Seyfried	CCM proteins modulate mechanotransduction via Klf2 during endocardial morphogenesis
12.00	Stanislao Igor Travisano	Endothelial Jag1/*Efnb2* signaling is essential for coronary vessel formation and myocardial compaction

12.15	Lunch, trade stands and odd poster presentations (12.45-1.30)

Session 4	**Coronary vessels and endocardium**	Chairs: Sarah Ivins and Jose Maria Perez-Pomares
13.45	Sarah Ivins	Coronary artery development and the role of CXCL12/CXCR4 signalling
14.15	Sophie Payne	Transcriptional regulation of coronary vessel growth during development and after injury
14.30	Catherine Roberts	Regulation of RA availability by Cyp26b1 in the developing heart is critical for normal patterning and vascularisation
14.45	Paul Palmquist-Gomes	A developmental model for the pathogenenesis of cardiac arterio-ventricular fistulae
15.00	Ghislaine Lioux	Outflow tract epicardial-like cells arise from the second heart field and generate various vascular cell types including part of the lymphatic cardiac vasculature

15.15	Coffee, trade stands and poster viewing

Session 5	Chairs: Stephane Zaffran and Bill Chaudhry	**Valves**
15.45	Stephane Zaffran	Development of the cardiac valves: Identification of a subpopulation of neural crest cells involved in aortic valve formation
16.15	Russell Norris	Cilia and Valvular Heart Disease
16.30	Donal MacGrogan	Signalling cross-talk during cardiac valve growth and morphogenesis: identification of Notch–Hbegf interacting pathways
16.45	Deborah Henderson	Novel sources of cells contribute to the arterial valve leaflets

17.00	Short break

17.10	Keynote Lecture: Professor Mary Herbert	Can IVF-based technologies prevent germline transmission of mitochondrial DNA disease?

18.00	Free evening in the city of Newcastle Upon Tyne

18.00	Nucleus business meeting	(WG-DAP nucleus members only)

Thursday 17th November: Great North Museum (Hancock)

Session 6	Chairs: David Brook and Deborah Henderson	**Genetics of human congenital heart defects**
9.00	David Brook	The genetics of human congenital heart disease
9.30	Duncan Sparrow	Environmental influences on mouse embryonic heart development
9.45	Lars Allen Larsen	Familial co-occurrence of congenital heart defects follows distinct patterns
10.00	Ahlam Alqatani	Optimal DNA extraction for NGS from archival formalin fixed tissues
10.15	Amina Kamar	A Novel Role for CSRP1 in a Lebanese Family with Both Cardiac Congenital Defects and Polydactyly
10.30	Melanie Phillipp	A new GRK5 loss-of-function variant in heterotaxy patients

10.45	Coffee

Session 7	Chairs: Thomas Brand and Maurice van den Hoff	**Conduction System**
11.15	Thomas Brand	A mutation in the cAMP-binding site of the Popeye domain containing 1 (POPDC1) gene causes atrioventricular block and limb-girdle muscular dystrophy (LGMD2X)
11.45	Silja Burkhard	Transcriptome analysis of the embryonic cardiac pacemaker domain in zebrafish
12.00	Caroline Choquet	Lineage analysis of ventricular trabeculae to decipher the role of Nkx2-5 in conduction system development

12.15	Lunch and even poster presentations (12.45-1.30)

Session 8	Chairs: Mathilda Mommersteeg and Marina Campione	**Myocardium and myocardial regeneration**
13.45	Mathilda Mommersteeg	Heart regeneration in the Mexican cavefish
14.15	Boudewijn Kruithof	A new *ex vivo* model to study cardiac fibrosis
14.30	Helen Phillips	Unraveling the underlying embryological origin of cardiomyopathy
14.45	Hector Sanchez-Iranzo	Transdifferentiation of cardiomyocytes during heart regeneration in the adult zebrafish
15.00	Fabien Kruse	Single-cell mRNA-sequencing of the regenerating zebrafish heart reveals distinct populations of cardiomyocytes

15.15	Prizes and closing remarks	Bill Chaudhry and Deborah Henderson


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Speaker abstracts

Tuesday 15th November

Outflow tract development: the regulation of second heart field progenitor cell deployment in the mouse

Robert G. Kelly

Aix Marseille University, CNRS UMR 7288, IBDM, Marseille, France

Here we will focus on early steps in development of the arterial pole of the mouse heart. The outflow tract is a hotspot of human congenital heart defects, as seen for example in 22q11.2 deletion (or DiGeorge) syndrome patients. The myocardial walls of the outflow tract are formed progressively by addition of progenitor cells from the second heart field (SHF). The SHF also contributes myocardium to the venous pole of the heart and is part of a larger field of cardiopharyngeal mesoderm that gives rise to craniofacial muscles. During heart tube extension SHF cells are located in the dorsal pericardial wall where they form an atypical epithelium. Direct or indirect perturbation of SHF development results in outflow tract shortening, abnormal ventriculoarterial alignment and a spectrum of common forms of congenital heart defects. Interactions with neural crest derived mesenchyme, that later drives separation of the outflow tract into the ascending aorta and pulmonary trunk, are essential for normal SHF deployment. Recent findings pertaining to the epithelial properties of SHF cells and the regulation of progenitor cell addition to the alternate poles of the heart will be presented, including the role of the 22q11.2 deletion syndrome candidate gene TBX1.

Cardiac looping is driven by heart tube-intrinsic morphogenesis in zebrafish

Federico Tessadori[(1)], Fabian K. Kruse[(1)], Susanne C. van den Brink[(1)] and Jeroen Bakkers [(1,2)]

[(1)] Hubrecht Institute-KNAW & UMC Utrecht, Utrecht, the Netherlands
[(2)] Interuniversity Cardiology Institute of the Netherlands, Utrecht, the Netherlands

During late somatogenesis, zebrafish cardiac progenitor cells coalesce at the embryonic midline to form the cardiac disc, which then rearranges into the linear heart tube. Concurrently, the cardiac tube extends leftward in a process called cardiac jogging. Throughout this process, cells originating from the left cardiac disc will mostly form the dorsal half of the cardiac tube, while right-originating cardiomyocytes compose the ventral half of the tube. Cardiac jogging is completed by 26 hours post fertilization. In the following 24 hours, the cardiac tube undergoes 3-dimensional rearrangements, referred to in their ensemble as cardiac looping, which result in the formation of a functional ventricle and atrium, separated by the atrioventricular canal (AVC). Although proper patterning and alignment of the cardiac segments is crucial to support the correct establishment of heart function, the process through which this is achieved remains ill-understood.
Current models in zebrafish suggest that at the end of cardiac jogging, the heart tube undergoes a rotation around its longitudinal axis, before undergoing looping morphogenesis. Through the use of transgenic fluorescent reporter lines we question here the accuracy of this model. During looping, cardiomyocytes in the forming ventricle and atrium rearrange in opposite directions towards the outer curvatures of the chambers, seemingly providing a torsion movement to the heart tube, around the AVC. To address which processes exert a major regulatory role in cardiac looping, we have tested various components of the process by assaying genetic patterning in hearts of zebrafish mutants displaying defective looping, assessing genetic interactions between major heart development regulators, manipulating cardiac looping by chemical treatment or *ex vivo* culture. We report here on our findings and discuss our model of a heart tube-intrinsic mechanism driving cardiac looping.

Cardiac looping may improve the efficiency of valveless pumping in the embryonic heart tube

Florian Hiermeier and **Jörg Männer**

Group Cardio-Embryology, Institute of Anatomy and Embryology, UMG, Georg-August-University Göttingen, Germany

The early embryonic heart is a tubular blood vessel, which generates unidirectional blood flow by a valveless pumping mechanism. At the present time, it unclear whether the embryonic heart tube works as a peristaltic pump or as a Liebau-effect pump. During the initial phase of its pumping activity, the originally straight heart tube is subjected to deforming forces that produce bending, twisting, kinking, and helical coiling of this specialized blood vessel. This deformation process is called cardiac looping. Its function is usually seen at late developmental stages. The prevailing view is that cardiac looping brings the segments of the embryonic heart tube and the developing great vessels into an approximation of their definitive topographical relationships. This should set the scene for the establishment of correct alignments of the pulmonary and systemic flow pathways in the mature, four-chambered heart of higher vertebrates. This idea, however, does not fit with the fact that cardiac looping is a phylogenetically highly conserved process among vertebrates. We postulate that the deformations caused by cardiac looping may improve the efficiency of valveless pumping in tubular blood vessels such as the embryonic heart tube. To test the physical plausibility of this hypothesis, we built a Liebau-effect pump and analyzed its pumping performance in a straight and a looped configuration over a range of compression frequencies corresponding to the physiological range of embryonic heart rates (0.5 - 3.0 Hz). The looped configuration was characterized by the presence of three kinks, which gave the pump a zig-zag course. We found that, compared to the straight configuration, the looped configuration significantly improved the pumping performance of our Liebau-effect pump. This effect was especially prominent when we increased the viscosity of the working fluid. These results confirm the physical plausibility of our idea that the looped configurations characterizing the tubular hearts of vertebrate embryos and of some basal chordates (tunicates) represent shapes that optimize the pumping function of this type of hearts. Future studies should clarify whether looped configurations may also increase the efficiency of valveless pumping in peristaltic pumps.

Detailed assessment of cardiac anatomy and microstructure in congenital heart disease by X-ray phase-contrast synchrotron radiation-based micro-CT

Patricia Garcia-Canadilla[1], Chong Zhang[1], Arjan J. Geers[1], Catalina Tobon-Gomez[1], Andrew C Cook[2], Bart Bijnens[1,3]

[1]PhySense, DTIC, Universitat Pompeu Fabra, Barcelona, Spain.
[2]UCL Institute of Cardiovascular Science, London, United Kingdom
[3]ICREA, Barcelona, Spain.

Background: Understanding the complexity of heart morphogenesis and the associated functional consequences of congenital heart disease (CHD) is essential for providing appropriate treatment strategies. Since our knowledge on the microstructure of the whole structurally abnormal heart in fetal & paediatric CHD is limited, novel imaging approaches offered by synchrotron facilities can provide structural detail currently not available otherwise. Our aim is to visualize and quantify cardiac ultrastructure in hearts with altered fetal development using X-ray phase-contrast synchrotron radiation-based micro-CT.

Methods: Two fetal hearts, one normal and one with Tetralogy of Fallot (TOF) of 15 weeks of gestation were selected from the UCL Cardiac Archive. While the specimens were kept in formalin, they were settled in water as supporting medium for acquisition. X-ray phase-contrast synchrotron radiation-based micro-CT was performed at 3.6μm resolution in Diamond Light Source, I13-2 beamline facilities. Several acquisitions were necessary to cover the whole heart along its long axis. The series were reconstructed using state-of-the-art filtered back projection.

Results: Fig 1 shows different image slices of both fetal hearts. The cardiac structures can be clearly differentiated (atria, ventricles, great vessels and valves). Myocyte orientation can be recognised as well in the ventricular walls (A-D). Fig.1E-F shows longitudinal reslices of both fetal hearts. The ventricular septal defect (D,F) as well as the overriding aorta (F) are clearly visible in the TOF fetal heart.

Conclusions: We managed for the first time to acquire μ-resolution microstructural datasets of whole human fetal hearts, illustrating normality as well as congenital malformations, resolving myofibre detail and providing information on cardiac microstructure without the need of slicing and sample processing.

Development and maldevelopment of the ventricular outflow tract

Robert H. Anderson
Cardiovascular Research Centre, Institute of Genetic Medicine, Newcastle University, Newcastle upon Tyne, UK

Kramer's account of the development of outflow tract was important for several reasons. In the first place, he pointed to the need to provide a readily understandable nomenclature for describing the complex temporal changes he observed. In the second place, he provided the first description of the intercalated cushions, showing how their inter-relationships with the distal ends of the fused major cushions produced the primordiums for formation of the arterial roots. His suggestion that the outflow tract itself could be described in bipartite fashion, however, and his introduction of the terms "truncus" and "conus", have yet to provide the clarity he sought, not least since he failed to indicate whether the arterial roots belonged to the so-called "truncus" or to the "conus". The availability of episcopic datasets from developing mice and human hearts now shows that, on the basis of the anatomical arrangement, it is possible to recognise three rather than two components of the developing outflow tract, with the solitary cavity of the tract becoming confluent with the cavity of the aortic sac at the margins of the pericardial cavity. When first formed, the outflow tract has exclusively muscular walls, and is lined by cardiac jelly. With ongoing development, and the ingrowth of non-myocardial walls from the second heart field, the distal myocardial border regresses from the margins of the pericardial border, with the non-myocardial tissues forming the parietal walls of the intrapericardial arterial trunks at the site of the initial distal component of the tripartite outflow tract. These developing trunks are separated distally by the intrapericardial growth of a protrusion from the dorsal wall of the aortic sac. As the protrusion, which is the primordial aortopulmonary septum, grows into the distal outflow tract, so the major cushions, which have developed in spiralling fashion by endothelial-to-mesenchymal transformation of the cardiac jelly, fuse to separate the intermediate part of the outflow tract, which along with the proximal outflow tract retains at this stage its myocardial walls. The protrusion then fuses with the distal margin of the major cushions, thus closing an embryonic aortopulmonary foramen. Failure to close this foramen results in postnatal persistence of an aorttopulmonary window.
By the time of closure of the aortopulmonary foramen, the intercalated cushions have been formed within the intermediate component of the outflow tract, thus setting the scene for formation of the arterial roots. At this stage, the major cushions in the proximal part of the outflow tract remain unfused. It is only at this stage, subsequent to separation of the intrapericardial aorta and pulmonary trunk, that it become possible to recognise the so-called "aortopulmonary septal complex". These columns, extending proximally within the unfused cushions, initially separate the arterial roots and ventricular outflow tracts, rather than the intrapericardial arterial trunks. With further development, the proximal cushions themselves fuse with each other, and also with the crest of the muscular ventricular septum, thus committing the aortic root to the developing left ventricle. The right ventricular surface of the fused proximal cushions then muscularises to form the subpulmonary infundibulum. The core of the fused cushions then attenuates in both the intermediate and proximal parts of the outflow tract. Eventually, therefore, there are no septal structures between any parts of the definitive right and left ventricular components of the initially solitary outflow tract. Complete failure of fusion of the major cushions underscores the postnatal persistence of a common arterial trunk, with the extent of formation of the aortopulmonary septum, and the extent of development of the intercalated cushions, accounting for the major morphological variants. Fusion of the major cushions, but failure of muscularisation of the proximal components, permits postnatal persistence of a doubly committed ventricular septal defect, which hence is closely related developmentally to common arterial trunk.

Morphology of ventricular outflow tract malformations

Vi –Hue Tran

Institute of Child Health, University College, London, UK

Understanding the anatomy of the normal heart in particular, the components of the right and left ventricular outflow tracts are crucial for the understanding of congenital heart defects. Tetralogy of Fallot and with pulmonary atresia, double outlet right ventricle, interrupted aortic arch, common arterial trunk and transposition of the great arteries are a group of congenital cardiac outflow tract malformations which are termed “conotruncal” anomalies. “Conotruncal” is not an appropriate term to use as the term “conus” and “truncus” will need to be defined. It is best to describe the phenotypic features as the morphologist/clinician sees it, as the embryology and development of some congenital heart defects are still uncertain. As the above congenital heart defects are a spectrum of disease, their anatomies will be reviewed separately.

Development of the (supra-) hepatic portion of the inferior caval vein

Jill PJM Hikspoors[1], Hayelom K Mekonen[1], Nutmethee Kruepunga[1], Mathijs MJP Peeters,[1] Greet MC Mommen[1], S Eleonore Köhler [1], Wouter H Lamers[1,21]

Department of Anatomy & Embryology, Maastricht University, Maastricht, The Netherlands

Background: Textbooks describe the (supra-) hepatic portion of the inferior caval vein as developing from the junctions of the distal portion of the vitelline veins with the venous sinus, while the distal portions of the umbilical veins would disappear without trace.

Methods: We investigated the (supra-) hepatic inferior caval vein in human and pig embryos between Carnegie Stages (CS) 10 and CS15, using Amira 3D reconstruction and Cinema 4D remodelling software.

Results: At CS10 the vitelline and umbilical veins joined the venous pole of the heart via a common trunk, the hepatocardiac channel, while the common cardinal veins only appeared at CS12. The liver bud began to expand into the transverse septum at CS12 and enveloped the vitelline veins at CS13. The vitelline veins remained identifiable as wide, dorsally bent conduits during CS13, with the right side being wider than the left. At this stage, the pig embryo differed from the human in that its liver consisted of a single ventromedial lobe overlying the gall bladder and two dorsolateral lobes containing the vitelline conduits. The expanding ventromedial liver lobe surrounded the umbilical veins during CS14. The umbilical veins remained identifiable as conduits up to the junction with the dorsolateral lobes, but the left vein was much wider than the right. The diameter of the right-sided venous sinus and the hepatocardiac channel that drained on it acquired a much larger diameter during CS14 than the corresponding left-sided structures, which become Marshall's ligament and disappear, respectively. The distal portions of both umbilical veins temporarily persisted in the lateral body wall through CS14 and continued to drain into the venous sinus via the hepatocardiac channels and became enveloped by the dorsolateral lobes during CS15. The portal vein now entered the right dorsolateral lobe via the duodenal inter-vitelline anastomosis and continued as the persisting dorsally bent conduit to the right hepatocardiac channel. At this stage, the portal sinus appeared as anastomosis between the right-sided vitelline conduit and the left-sided umbilical conduit.

Conclusions: The developmental transformation of the vitelline and umbilical veins proceeds according to a strict temporo-spatial plan. The embryonic pig liver has a more distinct bauplan than the human. Vitelline and umbilical veins develop ~2 CS earlier than the common cardinal veins and drain via hepatocardiac channels into the venous sinus. Left-right asymmetry in hepatocardiac channel development probably arises from the right-sided position of the sinoatrial junction and the decline of the left-sided portion of the venous sinus. Left-right anastomoses inside the liver develop at the junction of the ventromedial and dorsolateral lobes.

Morphological analysis suggests alternative classification of HLHS

Alqahtani A[1], Crucian A[2], Henderson D[1], O'Sullivan J[1,3], Brawn W[2], Anderson R[1] and **Chaudhry B[1]**

1. Cardiovascular Research Centre, Institute of Genetic Medicine Newcastle University, Newcastle upon Tyne, UK
2. Department of Cardiothoracic Surgery, Birmingham Children' Hospital, Birmingham, UK
3. Department of Paediatric Cardiology, Newcastle upon Tyne NHS Foundation Trust, UK

Background: Hypoplastic left heart syndrome (HLHS) is a rare congenital heart defect in which the left ventricle and aorta are severely underdeveloped; major repeated surgeries throughout childhood are required for survival. A spectrum of abnormalities involving stenosis or atresia of the mitral and aortic valves are also noted and there appears to be a strong genetic component to HLHS with up to 30% of relatives having either HLHS or a related heart malformation. Despite this, genetic studies have failed to reveal mutated genes that cause HLHS. We wondered if this failure to identify a shared genetic link is because HLHS is in fact a series of discretely different heart malformations that lead to common overall phenotype of hypoplastic left ventricle and ascending aorta.

Aim: We sought to objectively re-evaluate the morphology of HLHS and compare an alternative classification based on ventricular phenotype with the conventional clinical classification.

Methods: Segmental sequential analysis and physical measurements of HLHS hearts held in the historical surgical archive at Birmingham Children's Hospital, Birmingham, UK was carried out.

Results and Conclusions: This approach, based on ventricular and valvar morphology, suggested that at least three distinctly different core malformations exist. These findings suggest that the specific morphological appearances should be taken into account when analysing genomic data sets from patients with HLHS. Moreover, this study suggests that much of HLHS may be a developmental sequence.

Testing a Somatic Model of Hypoplastic Left Heart Syndrome

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Heart formation encompasses the highly orchestrated specification, differentiation, and morphologic patterning of a number of mesodermal- and endodermal-derived precursor cells within the developing embryo. Breakdown of the molecular networks that control these processes can result in congenital heart defects. Basic Helix-Loop-Helix (bHLH) transcription factors regulate numerous biological processes, and modulate transcriptional activity via the formation of dimers. The bHLH factor Hand1 is expressed within the myocardium developing left ventricle (LV) and the myocardial cuff (MC) between E8.5 and E13.5 in mice. Gene targeting models establish that Hand1 is required for normal LV development. Recently, a number of somatic mutations within the human *HAND1* gene have been associated with defects in the cardiac outflow tract [Wang, 2011], Hypoplastic Left Heart Syndrome (HLHS) [Reamon-Buettner, 2008], and intraventricular septum (IVS) defects. [Reamon-Buettner, 2009]. Intriguingly a *HAND1* frame shift mutation of A126fs was identified in 24 unrelated HLHS patient samples obtained from the Leipzig University collection. [Craatz, 2002] To validate this observation, we engineered a conditional *Hand1* allele (*Hand1*^{A126fs}) to examine the role this mutant protein in the development of HLHS. Preliminary results show that activation of *Hand1*^{A126fs} within the myocardium results in embryonic death due to defects in cardiac development. Phenotypes include poor compaction and IVS, extended OFT formation, and malformed hearts. Molecular analysis is underway to determine if this simulated somatic Hand1 mutation models HLHS.

Wednesday November 16th

Cardiac progenitors transcription program and heart defects

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The discovery of genes involved in heart development and congenital heart disease emphasizes the role of transcription factors in regulating expansion, fate determination and differentiation of cardiac progenitors.
Recent data have revealed that in the mouse, cardiac progenitors of the first and second heart fields (SHF) are specified during gastrulation from *Mesp1*-expressing cells. SHF progenitor cells, which are multipotent, later undergo self-renewal, morpho-dynamic changes, and differentiation. Among the transcription factors involved in these processes, Tbx1, encoded by a gene haploinsufficient in the DiGeorge/22q11.2 deletion syndrome, plays a critical role. Indeed, the loss of Tbx1 causes severe abnormalities of SHF-dependent cardiac structures, especially the outflow tract.

In this talk, I will present the latest data from the laboratory concerning the mechanisms by which Tbx1 performs its transcriptional functions. Tbx1 interacts with the SWI-SNF-like BAF chromatin remodeling complex as well as with the histone methyl transferase Mll3. These interactions lead to histone modifications, specifically H3K4me1, and possibly change in chromatin status, that is critical for Tbx1 functions. Drug-induced H3K4me1 enhancement ameliorates the consequences of Tbx1 loss of function suggesting that this modification is key in the transcriptional functions of this transcription factor. Bioinformatic analyses of genes associated with chromatin regions undergoing Tbx1-dependent chromatin modifications revealed genetic pathways critical in the biology cardiac progenitor cells.

Retinoic acid-dependent regulation of cardiac genes

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Defects in myocardial lineages of the venous pole of the heart can result in various congenital malformations such as misconnection of the pulmonary and caval veins, incomplete septation of atrial chambers and abnormal rhythms. Retinoic acid (RA) signalling is preferentially active in the venous pole of the embryonic heart. The formation of the venous pole depends on RA, the active derivative of vitamin A, by acting as a diffusible activator of nuclear receptors (RA receptors, RARs). The retinaldehyde dehydrogenase 2 (Raldh2) is responsible for RA synthesis during early development. Mutation of *Raldh2* results in embryonic lethality with venous pole patterning defects. Although there has been tremendous progress in characterizing the function of RA signaling during heart development, many gaps remain with respect to the underlying mechanisms of RA-mediated gene regulation. It has been very difficult to distinguish direct and indirect regulation of cardiac gene expression. Our understanding of the role of retinoids will be enhanced if such a distinction can be made for each regulated target gene. Moreover, frequent redundancy in receptor functions precluded their complete functional characterization. The aim of our project is to study in details how the critical RA signaling takes part in the regulatory mechanisms underlying the formation of the cardiac venous pole. In this study we have *used RNA- and ATAC*-sequencing approaches for profiling the transcriptome and open chromatin landscape of FACS sorted myocytes deficient for Raldh2 (Nkx2.5-GFP reporter) and Raldh2-expressing cells (Hoxb1-GFP reporter). An inducible conditional knockout mouse line of *Raldh2* is used to delete *Raldh2* in progenitor cells contributing to the venous pole (*Hoxb1-cre*). To identify the direct target genes of RARs in the developing heart, we determine the binding sites of RARa on a genome-wide scale by ChIP-seq from embryonic cardiac tissues. This study proposes to map RA response elements, which will be compared with loci associated with susceptibility to heart diseases.

***Tbx1* genetically interacts with *Pax9* to control fourth arch artery morphogenesis**

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The 22q11 deletion syndrome (22q11DS, also known as DiGeorge syndrome) is the commonest microdeletion syndrome in man, affecting 1 in 4000 births, and patients exhibit a wide variety of clinical abnormalities including cardiovascular defects. The transcription factor *TBX1* is the main candidate gene implicated in the aetiology of this syndrome. Complete loss of the *Tbx1* gene in transgenic mice results in common arterial trunk, a cardiovascular defect seen in 22q11DS patients. In *Tbx1*-heterozygous mice interrupted aortic arch is found, a phenotype also seen in 22q11DS patients, although this occurs in only a small percentage of mutant animals. Genetic analysis has revealed that the transcription factor *Pax9* is down-regulated in mice lacking *Tbx1. Pax9* is expressed at mid-embryogenesis in the pharyngeal endoderm and is co-localized with *Tbx1* expression. We have analysed embryos lacking *Pax9* and found that *Pax9* is required for correct cardiovascular development since all *Pax9*-null embryos exhibit a wide range of defects affecting the outflow tract and aortic arch arteries. To look for a genetic interaction *in vivo* between *Tbx1* and *Pax9* we attempted to generate double heterozygous mice but only two $Tbx1^{+/-};Pax9^{+/-}$ survived to weaning (33 were expected). Analysis of neonates and embryos revealed a statistically significant increase in the incidence of interruption of the aortic arch in $Tbx1^{+/-};Pax9^{+/-}$ mice, caused by a failure of the 4th pharyngeal arch artery to form. No evidence of a Tbx1:Pax9 protein-protein interaction was found, but preliminary data suggests that Tbx1 binds to the *Pax9* locus in chromatin immunoprecipitation and dual-luciferase assays. We speculate that fourth arch artery development is controlled via a *Tbx1-Pax9* genetic pathway and that *PAX9* may play a role in the pathogenesis of 22q11DS syndrome.

Whole exome sequencing identifies a novel candidate gene for congenital heart disease

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Congenital heart defects (CHD) are the most common type of birth malformation affecting 8 in 1000 live births. The etiology for these malformations remains unknown, but genetic factors play an important role. We identified a Danish family presenting autosomal dominant inherited CHD with reduced penetrance. Whole exome sequencing led to the identification of two missense mutations (p.Ser181Leu and p.Arg646Gln) encoded in the *PLEKHA6* gene. Both mutations segregate with CHD in the family, are not present in 2000 Danish controls, and display a very low frequency in the ExAC database of 64K exomes (MAF < 9.2e-06). *PLEKHA6* encodes a protein of unknown function, with gene ontology annotations related to phospholipid binding. The gene shows a high expression in embryonic hearts and is up-regulated during differentiation of stem cells into cardiomyocytes in the P19.CL6 stem cell model. Overexpression of GFP-tagged *Plekha6* in MDCK cells suggested localization of *Plekha6* to cell membranes. Mutant constructs are currently being analyzed.
Whole mount *in-situ* hybridization in 2 dpf wild-type zebrafish embryos shows *plekha6* expression in multiple organs and tissues, including brain, gut and heart. Surprisingly, knockdown of *Plekha6* in P19.CL6 cells increases the cells ability to differentiate into cardiomyocytes, suggesting an inhibitory function of *Plekha6* during cardiomyogenesis. We have performed *plekha6* gene knockdown and knockout in zebrafish embryos by injecting morpholinos and CRISPR-Cas9/guide RNA, respectively into fertilized oocytes. Depletion of *plekha6* results in cardiac defects (narrowed atrium inlet, misshaped heart and heart edema). CRIPSR-Cas9 mutated F0-fish were raised and out-crossed with wild-type lines, which resulted in 50% germline transmitted mutations in the F1 progeny and the founders were analyzed. Further proceedings with raising the next generation and the initial analysis of the mutants are ongoing.

Differentiation of hESC cells into the cardiac 'venous pole'/'cardiac inflow' cell types

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In order to study the molecular regulation of hESC differentiation into 'cardiac inflow cell types' we have used a NKX2.5-GFP hESC line, which shows endogenous reporter expression after NKX2.5 gene activation (Elliott et al., 2011). NKX2-5$^{eGFP/w}$ hESCs, aggregated as spin embryoid bodies (EBs), were induced to differentiate towards "proepicardial/epicardial-like" first by treatment with a cocktail of growth factors containing Activin-A, BMP4, Stem cell factor (SCF) and a GSK-3 b inhibitor-CHIR-99021 (days 0-3 of differentiation). EBs were treated with BMP4 from day 3 to day 4 of differentiation and EBs were treated with BMP4 + RA (1 mM) from day 4 to day 9 of differentiation. We analyzed the results obtained after culturing NKX2-5$^{eGFP/w}$ hESCs line cells with this different and specific conditioned media. This analysis was done with immunohistochemistry (IHC), quantitative PCR (RT-qPCR) and co-culture assay. We found that after this treatment, EB hESC expressed increased levels for genes such as RALDH2, COUP-TFII, Wt1, TBX5, TBX18, PDGFRa, and E-CADHERIN (RT-qPCR assessed), as compared to regular cardiomyocyte differentiation protocols. All these markers are characteristic of the 'cardiac inflow' (venous pole) of the heart (see Pérez-Pomares & de la Pompa, 2011), so it suggests that cells have acquired a "proepicardial/epicardial-like" phenotype *in vitro*. But since these genes are not exclusively expressed in epicardial progenitor cells, we further confirmed the functional proepicardial-like properties of RA + BMP4 induced cells using an in vitro co-culture assay and *in ovo* grafting of NKX2-5$^{eGFP/w}$ hESCs cells into the prospective pericardial cavity of developing chick embryos. Our results showed that NKX2-5$^{eGFP/w}$ hESCs cells can differentiate into embryonic epicardial progenitors and display functional properties of these cells (adhesion and spreading over the myocardium) following RA+BMP4 treatment. These data demonstrate that NKX2-5$^{eGFP/w}$ hESCs cells can be specifically differentiated into epicardial progenitor-like lateral mesodermal cells showing transcriptional and functional properties of their *in vivo* counterpart.

Coordination of Collective Cell Migration in Angiogenesis

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The endothelial lining of the vascular system is remarkably heterogeneous in its structure, function and developmental origins. In this session we will hear more about the diverse mechanisms that give rise to this heterogeneity during lymphatic, endocardial and coronary vessel morphogenesis. In each case, endothelial cells must communicate and coordinate their behaviour to collectively generate these diverse vascular tissues. My lab seeks to uncover how both cellular and multicellular polarity are established and maintained during nascent blood vessel formation to ensure that the collective movement of endothelial cells remains synchronised. In particular, we combine zebrafish *in vivo* experimental and *in silico* computational approaches to reveal that heterogeneity introduced by asymmetric cell division generates the multicellular polarity that drives coordinated collective cell migration in angiogenesis. I will discuss how asymmetric positioning of the mitotic spindle during endothelial cell division generates daughters of distinct size with discrete thresholds of pro-migratory Vegfr signaling. Consequently, post-mitotic Vegfr asymmetry drives the intrinsic self-organisation of daughters into leading and trailing collectively migrating cells. Considering that robust maintenance of leader-follower hierarchies is a hallmark of all collectively migrating cell systems, asymmetric division may drive growth of many tissues during development and regeneration.

Transcriptional control in the developing heart circulation

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The heart circulation emerges from the remodeling of paired embryonic arteries that run through the branchial arches and connect the heart to the dorsal aorta. To understand this process, we focused on the period when this embryonic area is being remodeled into the mature heart circulation. We examined: (i) global transcriptomes, (ii) chromatin modifications and (iii) binding of transcription factors in the posterior branchial arches (3-4-6) and in the outflow tract of mouse and human embryos. Our integrative analysis uncovered a distinctive smooth muscle cell-specific expression signature in the posterior branchial arches and the outflow tract. Moreover, we found that generation of vascular smooth cells, which is essential to the remodeling of the embryonic circulation, was specifically restricted to this embryonic area in vivo by the asymmetric distribution of a key transcriptional regulator. Finally, we identified non-coding regions of the genome that are active during remodeling of the human heart circulation, and whose genetic variants can lead to congenital heart disorders. Validating our strategy, the majority of these regions were sufficient to drive GFP expression in the heart region of zebrafish embryos. These findings cast light on how non-coding variations identified in genome-wide association studies (GWAS) contribute to biological mechanisms and disease.

CCM proteins modulate mechanotransduction via Klf2 during endocardial morphogenesis

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Endocardial cells are specialized endothelial cells that line the interior of the heart tube. With the assembly of a heart tube and the onset of blood flows, endocardial cells are exposed to different hemodynamic forces including frictional laminar wall shear stress, retrograde oscillatory flows, and radial wall pressure. In response to these biophysical forces, endocardial cells activate mechanosensitive signaling which results in a number of cellular adaptations thereby contributing to the morphogenesis of the heart. The best characterized mechanosensitive gene within endothelial cells is the zinc finger transcription factor Krüppel like factor 2 (Klf2). Among the blood flow-sensitive developmental processes that are regulated by Klf2 is the remodeling of endocardial cushions into functional cardiac valve leaflets.

The mode by which the mechanosensitive Klf2 signaling pathway within the endocardium is regulated has remained largely unknown. Recent studies in mouse and zebrafish revealed that proteins of the cerebral cavernous malformations (CCM) complex control expression levels of Klf2 (Renz et al., 2015 Dev. Cell 32, 181-190; Zhou et al., 2015 Dev. Cell 32, 168-180; Zhou et al., 2016 Nature 532, 122-126). Here, we will summarize new data on the physiological roles of CCM proteins in controlling Klf2 activity within the endocardium and will also consider how the deregulation of blood-flow-responsive endocardial Klf2 signaling can result in pathophysiological changes within the heart.

Endothelial Jag1/*Efnb2* signaling is essential for coronary vessel formation and myocardial compaction

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Notch signaling plays critical roles in vertebrate cardiac development and is required for the formation of ventricular chambers, cardiac valves and coronary vessels. We examined the role of Notch ligand Jagged1 (Jag1) during coronary vessels development. Jag1 is expressed in the sinus venosus (SV) at E11.5, and later in developing coronary vessels. Endothelial/endocardial-specific deletion of *Jag1* using the *Nfatc1-Cre* driver line caused valves enlargement, thin compact myocardium, decreased myocardial proliferation, defective coronary arteries formation and lethality at E13.5. Affected coronaries showed reduced vessel branching from the SV and reduced Notch1 activity in emerging arteries with concomitant reduced expression of downstream targets such as *Efnb2*. We further examined the role of Ephrin B2 as a downstream Notch effector in coronary development. *Efnb2^{flox};Nfatc1-Cre* mutants displayed defective coronary development, characterized by increased vessels density resulting in the aberrant assembly of the coronary network, reduced myocardial compact zone and muscular ventricular septal defects. Notch pathway activity was increased suggesting a positive feedback loop between *Efnb2* and Notch. In E11.5 vascular endothelial growth factor-treated ventricular explant assays, vessel growth and plexus remodeling was markedly reduced in both *Jag1* and *Efnb2* mutants but increased in *Dll4^{flox};Nfatc1-Cre* and *Notch1^{flox};Nfatc1-Cre* mutants, suggesting that Jag1 function in coronary artery formation is mediated, at least in part, by *Efnb2*. These data support the existence of a vascular endothelium hierarchical network involving Jag1/Notch1 signaling upstream of *Efnb2,* and suggest that myocardial compaction and coronary vessel development are intimately-linked processes in which *Efnb2* plays a key role.

Coronary artery development and the role of CXCL12/CXCR4 signalling

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Coronary artery disease leading to myocardial infarction and heart failure is a major cause of death globally. Improving our understanding of the molecules and processes involved in coronary vessel development is important to help in the identification of possible targets for future therapeutic interventions aimed at re-vascularising injured hearts.
Identifying the source from which coronary endothelial cells (ECs) originate has been the subject of numerous studies and a certain amount of controversy in the last few years. The latest data from lineage tracing experiments will be discussed, as well as the morphological events underlying the development of a mature coronary plexus. Formation of the coronary stems is a critical step in this process, which depends on the establishment of anastomotic connections between the peritruncal capillary network and the lumen of the developing aorta. We have shown that in mice deficient for the chemokine CXCL12 the formation of such connections is disrupted leading to severe coronary artery defects. Its receptor CXCR4 is expressed in peritruncal ECs as well as in arterial-fated, myocardially- localised ECs. The transcriptional profile of CXCR4-expressing coronary ECs is now being analysed in order to understand the signalling pathways involved in the early stages of coronary arteriogenesis.

Transcriptional regulation of coronary vessel growth during development and after injury

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Whilst recent research demonstrates that coronary endothelial cells are developmentally heterogeneous with multiple origins, the molecular regulation of coronary vessel growth remains incompletely understood. We aim to interrogate the multiple upstream pathways regulating the formation of coronary vessels in both development and in a murine model of myocardial infarction through the study and manipulation of well-characterized vascular enhancers.

Gene enhancers are densely clustered groups of transcription factor binding motifs, and are the primary regulators of tissue-specific gene transcription. We have previously identified endothelial-specific enhancers activated by multiple independent transcriptional pathways. These enhancers, when fused to the LacZ reporter gene, can now provide crucial information about the role of these transcriptional networks in development and disease.

Using these enhancers, we now have evidence of at least two different regulatory cascades at play during embryonic coronary vessel development. Fascinatingly, although these regulatory cascades concur with previously described sinus-venosus and endocardial origins for coronary vessels during embryonic development, our preliminary experiments using myocardial infarction challenge the current understanding of coronary origins in the postnatal heart. This work therefore provides both independent evidence of heterogeneity within the coronary endothelium, and a novel tool to investigate postnatal and pathological coronary formation.

Regulation of RA availability by Cyp26b1 in the developing heart is critical for normal patterning and vascularisation

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Gradients of the morphogen retinoic acid (RA) across developing tissues produce specific transcriptional read-outs leading to the proper patterning of many body systems, including the heart. Cytochrome p450 (CYP) enzymes metabolise biologically active molecules, with the CYP26 sub-family having a high affinity for RA. Expression of CYP26 enzymes is necessary to protect tissues against inappropriate RA signalling and to provide RA "sink" regions, to counterbalance regions of retinaldehyde dehydrogenase 2 (RALDH2)-mediated synthesis of RA , thus establishing RA gradients across tissues. Expression of *Cyp26b1* in developing cardio-pharyngeal regions is necessary to properly pattern the vertebrate heart. In the absence of *Cyp26b1* expression, both arterial and venous pole development are abnormal. The former gives rise to great vessel and outflow tract phenotypes reminiscent of 22q11 Deletion Syndrome, which are synergistic with additional loss of *Tbx1* alleles. The latter produces abnormal epicardial development causing loss of coronary vessels, failure of myocardial compaction and mid-gestational death in 90% of mutant embryos. Coronary stems and the ventricular coronary plexus are affected, failing to grow and connect with each other. Surface epicardial cysts connected directly to the ventricular lumen are formed, whilst vascular endothelial marker expression is reduced, with coronary artery development particularly disrupted. Current evidence suggests that epithelial-mesenchymal transition (EMT) abnormalities may underlie these defects. Reduced numbers of epicardial cells initiate EMT. Those cells which are positive have diminished ability to migrate into the myocardium. Ectopic surface differentiation of those cells still able to initiate EMT likely produces epicardial vascular cysts. The resulting loss of epicardially-derived-cells probably contributes to the coronary vascular defects. We are currently exploring whether the reduced coronary endothelial network is solely the result of aberrant epicardial signalling and can be rescued, or if autonomous defects of sinus venosus development may also contribute. *Cyp26b1* mediated regulation of RA-signalling is also necessary for proper partitioning of the heart, as a strongly penetrant atrioventricular septal defect has been identified. Finally, the cardiac valves do not develop normally. Thus regulation of RA availability by *Cyp26b1* in the developing heart is critical for normal patterning and vascularisation.

A developmental model for the pathogenenesis of cardiac arterio-ventricular fistulae

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Coronary Artery Fistulae (CAF) are congenital coronary artery (CA) anomalies consisting of an abnormal communication of a coronary artery with either a cardiac chamber or a large cardiac vessel. Although their incidence in the Western population is low, CAF can lead to complications such as myocardial hypertrophy, endocarditis, heart dilatation and cardiac failure. CAFs can appear as an isolated anomaly or linked to some other forms of congenital heart disease like Left Ventricular Non-Compaction (LVNC) and intrinsic CA anatomy anomalies, but their etiology remains unknown. In this work we have used two different experimental models (transgenic mice and avian embryos) to investigate on the developmental mechanics of CAF formation. In order to tackle this goal, we have manipulated epicardial development and ventricular wall compaction, two inextricably related developmental events during coronary embryogenesis. Conditional integrin a4 gene deletion in the septum transversum/proepicardial (ST/PE) region (G2-Gata4+) disrupts early epicardium development and reduces cardiomyocyte proliferation, leading to the thinning of the ventricular compact myocardial layer. Reduction in compact myocardium thickness associates to the presence of multiple ventricular myocardial discontinuities and focal endocardial extrusion. This same phenotype can be experimentally reproduced in chick embryos using a cryocauterization method (Palmquist-Gomes et al., 2016). Our results suggest that the partial absence of epicardium in a4integrin;G2-Gata4Cre mouse embryos and the cryoinjury in avian embryos generate myocardial discontinuities in the embryonic ventricular wall, which promote endocardial extrusion towards the pericardial cavity and the early contact of the endocardium with coronary progenitors at the epicardial surface of the heart. In the case of avian embryos, this phenomenon leads to precocious smooth muscle differentiation from epicardial mesenchymal cells, and the formation of pouch-like structures that closely resemble CAF. We conclude that anomalous compact myocardial embryonic growth can originate CAF.

Outflow tract epicardial-like cells arise from the second heart field and generate various vascular cell types including part of the lymphatic cardiac vasculature

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During embryonic development, the outflow tract (OFT) connects the heart chambers with the systemic and pulmonary circulation. The OFT is composed of endothelial cells, smooth muscle and fibroblasts known to derive from neural crest and second heart field (SHF) progenitors. The OFT is also covered by a mesothelial sheet of arterial epicardial-like cells (aELCs) continuous with the cardiac outermost layer, the epicardium. While the epicardium arises from the proepicardium, aELCs are derived from the cephalic pericardium but their exact origin and function remain unclear. Performing random retrospective clonal analysis we show the existence of a common multipotential progenitor cell (MPC) contributing to all OFT layers including aELCs and part of the ventricular lymphatic vasculature. We sought to address two questions: (1) if clonal groups identified are derived from the second heart field and (2) if aELCs represent the MPCs contributing to all OFT layers and lymphatic cells. Genetic lineage tracing using SHF as well as Mesothelial tissue-specific cre lines recapitulated labelling of all OFT layers and lymphatic cells, consistent with data obtained by clonal analysis. As a result, we propose a model in which MPCs from the second heart field generate a mesothelial intermediary, aELC, which in turn contributes to the OFT layers and to the ventricular lymphatic vasculature. We are currently performing RNA seq comparing dissected embryonic aELCs with epicardial cells to appreciate their distinct nature and cell fates. Lower protein levels of Raldh2 in aELCs compared to epicardial cells might point to a key role for Retinoic acid signalling in the specification of aELC.

Development of the cardiac valves: Identification of a subpopulation of neural crest cells involved in aortic valve formation

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With a worldwide incidence of 1-2%, valve disease is one of the predominant causes of cardiovascular morbidity and mortality in all age groups. Valve defects are present in a quarter of all congenital heart diseases. In addition, these defects can occur as a feature of multisystemic rare diseases such as Marfan, Turner, Williams, Loeys-Dietz, Ehlers-Danlos, and Noonan syndromes. The cellular mechanisms that underlie cardiac valve development are poorly understood. Valve leaflets originate from endocardial cushions, which are populated by cells derived from the endothelium. Cushion formation is followed by a process of excavation that takes place at the distal end of each cushion, thinning the valve tissue thus creating its final shape. Semilunar valve development is distinguished from atrioventricular valve formation by the colonization of outflow tract cushions by neural crest-derived cells. A recent study has shown that neural crest cells play essential roles in positioning the cushions, and patterning the valve leaflets. Using genetic lineage analysis we identified a sub-population of neural crest cells that contribute to semilunar valve formation. These neural crest-derived cells are preferentially located at the interleaflet triangle of the valve leaflets. Here we show how alterations of this specific population affect aortic valve development leading essentially to a bicuspid aortic valve.

Cilia and Valvular Heart Disease

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Mitral valve prolapse (MVP) affects 2.4% of the population and is the most common cause for mitral valve surgery. MVP is characterized by excessive mitral valve tissue growth resulting in prolapse, impaired valve closure and mitral regurgitation (MR). Serious complications resulting from valve dysfunction include endocarditis, congestive heart failure, and sudden cardiac death. Familial and genome wide population studies by our group have demonstrated that MVP can manifest during childhood, suggesting that genetic abnormalities expressed during valve development become exacerbated over a lifetime. Here we report the discovery of multiple mutations in a cilia-related gene that segregates with non-syndromic MVP in multiple families. Loss of function mouse models fully recapitulated the structural and functional aspects of MVP observed in the families. We traced the inception of disease to developmental defects in cilia signaling. Two hybrid screens identified novel ciliary interacting proteins and major signaling factors that regulate valve progenitor cell differentiation. The importance of cilia during development was further validated by genetically ablating the ciliary axoneme in vivo. These cilia-deficient mice fully recapitulated the structural and functional defects that define MVP. These studies delineate the importance of primary cilia function in mitral valve development and define, for the first time, that novel mutations in a cilia gene cause MVP in humans.

Signalling cross-talk during cardiac valve growth and morphogenesis: identification of Notch–Hbegf interacting pathways

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Rationale: The Notch signalling pathway is crucial for cardiac valve formation. Mice lacking the Notch ligand Jag1 or Notch1 receptor in endocardium (*Jag1flox; Nkx2.5-Cre*, *Jag1flox;Nfatc1-enCre* or *Notch1flox;Nfatc1-enCre* mutants) have enlarged valve cusps, bicuspid aortic valve, and septal defects, indicating that Jag1 to Notch1 signalling is required for proper post–epithelial–mesenchymal transition valvulogenesis. Downstream of Notch, Hbegf is required to restrict mesenchyme proliferation and valve growth.
Objective: Determine interacting pathways upstream of Jag1-Notch1-Hbegf signalling during valvulogenesis.

Methods and Results. We performed global gene expression analysis of wildtype and *Jag1flox; Nkx2.5-Cre* valves at E14.5. Functional analysis of the differentially expressed genes identified several relevant Gene Ontology (GO) categories, including for general morphogenetic processes such as aggregation, regionalization, cell proliferation, cell adhesion, and cellular component movement. Highly enriched GO terms more specifically related to valve development were collagen fibril organization, heart valve morphogenesis, smooth muscle cell differentiation, and regulation of ossification. Ingenuity Pathway Analysis further identified >15 altered pathways, including for developmental signaling, such as BMP and Ephrin receptor pathways that have been linked to valve development. Signaling and activation hallmarks acting upstream (CXCR4, thrombin, and cardiac–adrenergic) and downstream (c-AMP, calcium, Gαi proteins, and phospholipase C) of G-protein–coupled receptors were also highly perturbed. G-protein–coupled receptor signaling notably through the Cxcl12/ Cxcr4 pathway was significantly downregulated. Arterial valve defects similar to those seen in endocardial Notch mutants have been observed in Cxcl12 or Cxcr4 mutants suggesting a functional link between Notch and Cxcl12-Cxcr4 pathway in cardiac valve development.

Conclusions: Differential gene expression analysis of *Jag1flox; Nkx2.5-Cre* mutant valves identified multiple altered processes and signaling pathways relevant to valve development. We are currently evaluating the functional significance of the Cxcl12-Cxcr4 pathway in the origin of congenital heart defects associated with reduced NOTCH function.

Novel sources of cells contribute to the arterial valve leaflets

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Background: Abnormalities of the valves of the heart, including bicuspid aortic valve (BAV) are some of the commonest congenital malformations. The cardiac valves are derived from embryonic precursors known as endocardial cushions. These cushions are populated by cells derived from the endocardium (EDC), and in the case of the arterial valves, also by neural crest cells (NCC). Almost nothing is known, however, about an additional set of "intercalated cushions" found in the outflow tract, that also contribute to the arterial valves.

Aim: To investigate the origin of the intercalated cushions, and how these contribute to the arterial valve leaflets.

Methods: The development of the arterial valve leaflets was analysed using immunohistochemistry and Cre-lox based lineage tracing, together with the Vangl2$^{f/f}$ mouse line.

Results and Discussion: The intercalated cushions first appear as swellings within the outflow wall at E10.5 at the same time as the main outflow cushions are forming. Immunohistochemical analysis showed that at the earliest stages, the intercalated cushions do not express typical cushion markers and appear to form by a different mechanism to the main cushions. Cre-Lox based lineage tracing revealed that EDC and NCC make a minor contribution to the intercalated cushions. We show that Mef2c-AHF-Cre labelled second heart (SHF) cells that have not passed through the endocardial lineage, form the intercalated cushions. These SHF-derived cells also express cardiac TroponinT-Cre, but do not express other myocardial markers indicting they remain incompletely undifferentiated prior to forming the intercalated cushions. Analysis of Vangl2;Isl1Cre mutant embryos indicated that the planar cell polarity gene Vangl2 is required in SHF progenitors for normal formation of the intercalated cushions. Disruption of intercalated cushion formation can result in BAV, suggesting a novel mechanism for the development of this common malformation.

Keynote Presentation: Professor Mary Herbert
Professor of Reproductive Biology

Can IVF-based technologies prevent germline transmission of mitochondrial DNA disease?

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Mitochondrial DNA (mtDNA) mutations are maternally inherited and are associated with a broad range of debilitating and fatal diseases. Reproductive technologies designed to uncouple the inheritance of mtDNA from nuclear DNA may enable affected women to have a genetically related child with a greatly reduced risk of mtDNA disease. To this end, we have performed preclinical studies to test the safety and efficacy of pronuclear transplantation (PNT). Surprisingly, techniques developed using abnormally fertilized human zygotes are not well tolerated by those that undergo normal fertilization. We have therefore developed an alternative approach based on transplanting pronuclei shortly after completion of meiosis rather than shortly before entry into the 1st mitotic division. We find that this early PNT (ePNT) technique promotes efficient development to the blastocyst stage with no detectable effect on aneuploidy or gene expression. However the efficacy of ePNT in preventing transmission of mtDNA disease also depends on minimising co-transfer of mtDNA during pronclear transplantation. Following optimisation of procedures, mtDNA carryover was <2% in the majority of ePNT blastocysts. However, ~20% of hESC lines derived from ePNT blastocysts showed an increase in heteroplasmy due to mtDNA carryover. While the relevance of this to development *in vivo* is unclear, the finding underscores the importance of reducing mtDNA carryover to the lowest possible levels. We propose that ePNT has the potential to give rise to normal pregnancies with a reduced risk of mtDNA disease. Our ongoing work is focused on further reducing this risk by minimizing mtDNA carryover.

Mary Herbert is Professor of Reproductive Biology at Newcastle University. She is also an Honorary Consultant Clinical Embryologist at Newcastle Fertility Centre. She leads a team of clinical and research scientists working side by side on a program of basic and clinical research. Her group's research is currently focused on (i) Developing IVF-based techniques to prevent transmission of mitochondrial DNA disease (ii) Understanding the molecular basis for female age-related infertility

Thursday 17th November

The genetics of human congenital heart disease

David Brook

University of Nottingham, UK

Heart disorders are the most form of congenital abnormality and affect around 1 in 150 newborns. Initial studies to identify the underlying genetic causes involved pedigree analysis for Mendelian forms of congenital heart disease (CHD) and mutation studies of single genes, which were often directed by knowledge acquired from mouse genetics. Recently there has been a rapid increase in our understanding of the genetics of CHD as a result of Nextgen sequencing of CHD cases and controls. This talk will provide an overview of recent data and an introduction to Session 6: Genetics of human congenital heart disease.

Environmental influences on mouse embryonic heart development

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University of Oxford, UK

Congenital heart disease (CHD) is the most common human birth defect, affecting ~1% of live births worldwide. However, despite intensive investigation by classical and next-generation genetic and genomic methods, only ~20% of cases can be explained genetically. This is because environmental stressors can also cause CHD. Such factors have been identified using epidemiology or animal studies, and examples include maternal conditions and diseases such as viral infection and hyperthermia, folate deficiency, hypertension, stress, pre-existing diabetes and phenylketonuria. In addition, maternal exposures to pharmaceuticals such as anti-convulsant and anti-arrhythmia ion-channel blockers, anti-depressants, retinoic acid, thalidomide or environmental pollutants have also been associated with increased CHD risk. Despite these factors being known for decades, virtually nothing is known of how they cause CHD. We have developed mouse models of several of these environmental factors to elucidate the molecular mechanisms by which they perturb embryonic heart development.

Familial co-occurrence of congenital heart defects follows distinct patterns

Ellesøe SG [1], Workman CT [2], Bouvagnet P [3], Loffredo CA [4]; McBride KL [5], Hinton RB [6]; van Engelen K [7], Gertsen EC [7], Mulder BJM [8], Postma AV [7, 9], Anderson RH [10], Hjortdal VE [11], Brunak S [1], **Larsen LA[12]**

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Congenital heart defects (CHD) affect almost 1% of all live born children and the number of adults with CHD is increasing. In families where CHD has occurred previously, estimates of recurrence risk and the type of recurring heart defect are important for counseling and clinical decision making, but the recurrence patterns of CHD in families are poorly understood. We aimed to determine recurrence patterns in CHD, by investigating the co-occurrences of congenital heart defects in 1,163 CHD families, comprising 10,278 individuals, of which 3,080 had a clinically confirmed CHD diagnosis. We calculated rates of concordance and discordance for 42 types of CHD, observing a high variability in the rates of concordance and discordance. By calculating Odds Ratios for each of 1,771 pairs of discordant lesions observed between affected family members, we were able to identify 192 pairs of malformations that co-occurred significantly more or less often than expected in families. The data show that distinct groups of cardiac malformations co-occur in families, suggesting influence from underlying developmental mechanisms. Analysis of data from mouse models with malformations corresponding to familial CHDs showed that susceptibility genes were shared in 21.4% of pairs of co-occurring discordant malformations but in none of malformations that rarely co-occur, suggesting that a significant proportion of co-occurring CHD in families is caused by overlapping susceptibility genes. In conclusion, our data show that familial CHD follow specific patterns of recurrence and suggest that part of co-occurrence of malformations in familial CHD may be caused by shared susceptibility genes.

DNA extraction from formalin fixed tissues

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Background: Historical collections of human hearts with congenital malformation are an important resource for understanding morphology. Similarly, in familial congenital heart disease, there may be historical tissue samples that could be used to confirm the presence of particular mutations throughout a family pedigree. However, there are concerns regarding the use of these samples in genomic studies because of formalin fixation. Formalin not only forms covalent bonds between DNA strands, but also links proteins to the double helix reducing the efficiency of the polymerase chain reaction (PCR). In addition, formalin induces DNA breakage and chemical modifications to bases that prevent successful Sanger sequencing and also produce erroneous sequencing. Next generation sequencing (NGS) of exomes requires short DNA fragments and may overcome difficulties of traditional Sanger sequencing.

Aim: We sought to develop a cost effective method to extract high quality DNA from formalin fixed tissue samples that would be suitable for analysis by NGS using the Illumina MiSeq sequencing platform and would also allow confirmation of findings by PCR and conventional Sanger sequencing.

Method: human adult artery from surgical specimens, stored in formalin for over nine months, was provided by the Department of Pathology, Newcastle Hospitals NHS Foundation Trust. We initially isolated genomic DNA from this using classical proteinase K digestion followed by phenol:cholorform extraction and analysed the yield, purity, fragment length and suitability for PCR. We then modified the protocol, investigating the effect of temperature and chelating resin on the extraction process. We then compared our optimised methods with GeneRead™ DNA FFPE Kit (Qiagen). A column-based commercial DNA extraction kit that also reverses formalin amination.

Results and conclusion: We were able to demonstrate a DNA extraction protocol which produced large amounts of genomic DNA with quality that was at least as good as commercial kit based techniques in terms of DNA fragment size and PCR behaviour. However, in assessing the behaviour of the extracted DNA we demonstrate corruption of sequencing results at frequencies that may masquerade as a single nucleotide polymorphism.

A Novel Role for CSRP1 in a Lebanese Family with Both Cardiac Congenital Defects and Polydactyly

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Background: Abnormal cardiac development leads to human congenital heart disease (CHD), which is responsible for the vast majority of neonates' death around the world. DHFMR-85 is a Lebanese family composed from the consanguineous marriage between 2 first-degree cousins. Out of the 7 conceived children, 2 died *in utero* at the ages of 6 and 9 months of unknown causes. Of the remaining 5 children, 3 have congenital heart disease (ventricular septal defect, atrial septal defect, and patent ductus arteriosus), and 4 have polydactyly.

Methods and Results: Targeted exome sequencing identified a heterozygote duplication of a 14 nuc fragment in exon 5 of *CSRP1*, causing a frameshift mutation at position 154 of the protein. Genotyping all family members with Sanger sequencing showed that this mutation is segregating with the CHD phenotype but not with polydactyly except for the father who has no cardiac problems and yet is positive for the mutation. The variant was not found in 200 exomes of Lebanese origin. *CSRP1* encodes a LIM domain protein, shown to be implicated in smooth muscle function. The E154fs mutation totally disrupts the second LIM domain. Immunohistochemistry on cardiac tissues from mouse embryos at E14.5 reveals the endogenous expression of CSRP1 in mammalian heart. In addition, immune-staining done on Hela cells shows that E154fs mutation did not affect the cellular localization of the protein and both the WT and E154fs CSRP1 shuttle between the cytoplasm and nucleus. In addition, by luciferase assay we show that the WT CSRP1 activates the transcription of cardiac developmental promoters VEGF, NOS3 and NPPA. However, the E154fs CSRP1 variant significantly abrogates this activation. In addition, it differentially inhibits the physical association of CSRP1 with SRF and GATA4.

Conclusion: The *in vivo* expression of CSRP1 in heart confirms its potential role in cardiac development and the novel mutation detected in the Lebanese family with various forms of CHD is disease causing. This new finding provides fundamental knowledge into the molecular basis of congenital heart defects and could unravel additional pathways involved in cardiac development.

A new GRK5 loss-of-function variant in heterotaxy patients

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In the adult organism, G protein-coupled receptor kinases such as GRK2 and GRK5 represent crucial regulators of cardiac performance. They are thus considered potential therapeutic targets in heart failure. Interestingly, GRK5 is already expressed during development and we have previously uncovered that GRK5 determines left-right asymmetry and proper heart development. In the current study we wanted to identify GRK5 variants of functional significance by analysing 187 individuals with laterality defects (heterotaxy) that were associated with a congenital heart defect (CHD). Using conventional Sanger sequencing of exons we identified two moderately frequent variants in GRK5 with minor allele frequencies <10%, and seven very rare polymorphisms with minor allele frequencies <1%, two of which are novel variants. Given their evolutionarily conserved position in zebrafish, we performed in-depth functional characterisation of four variants (p.Q41L, p.G298S, p.R304C and p.T425M). In detail, we tested the effects of these variants on normal subcellular localisation and the ability to desensitise receptor signalling. Most importantly, we also assessed the variants' ability to correct the left-right asymmetry defect upon Grk5l knockdown in zebrafish. p.Q41L, p.R304C and p.T425M responded normally in the first two aspects, however, neither p.Q41L nor p.R304C were capable of rescuing the lateralisation phenotype. The fourth variant, p.G298S is likely to destabilize the conformation of GRK5 and could be identified as a complete loss-of-function variant in all assays. We therefore conclude that GRK5 p.G298S may predispose to the development of heterotaxy-associated CHD.

A mutation in the cAMP-binding site of the Popeye domain containing 1 (POPDC1) gene causes atrioventricular block and limb-girdle muscular dystrophy (LGMD2X)

Thomas Brand

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The Popeye domain containing (POPDC) gene family encodes membrane proteins with an intracellular Popeye domain, which acts as a high-affinity cAMP-binding domain. Three POPDC genes are present in vertebrates, which display an overlapping expression pattern and are abundantly expressed in striated muscle tissue. In animal models, *Popdc1* acts an essential regulator of structure and function in the heart and skeletal muscle. However, POPDC1 mutations have not been associated with human disease. Here, we describe a homozygous missense variant (c.602C>T, p.S201F) in POPDC1, identified by whole-exome sequencing, in a family with cardiac arrhythmia atrioventricular block and limb-girdle muscular dystrophy (LGMD). This allele segregated with the pathological phenotype in this family. It was absent in databases and was not found in 104 patients with a similar phenotype, suggesting this mutation to be family-specific. Compared with WT protein, POPDC1S201F displayed a 50% reduction in cAMP affinity. Muscle biopsies of affected family members revealed a reduction in plasma membrane localisation of the mutant POPDC1 protein and of POPDC2. In Xenopus oocytes, WT but not the mutant protein caused an enhanced membrane trafficking of the two-pore-domain potassium channel TREK-1. Aberrant TREK-1 gating properties were also observed in the presence of the POPDC1S201F mutant. Consistent with these findings, forced expression of POPDC1S201F in a murine cardiac muscle cell line (HL-1) increased hyperpolarization and upstroke velocity of the action potential. Zebrafish mutants carrying the homologous mutation, popdc1S191F, displayed heart and skeletal muscle phenotypes that resembled those observed in patients. Our study therefore identifies POPDC1 as a novel disease gene causing a very rare autosomal recessive cardiac arrhythmia and LGMD.

Transcriptome analysis of the embryonic cardiac pacemaker domain in zebrafish

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Rhythmic contraction of the developing zebrafish heart can be observed as early as 24 hours post fertilization. The initial peristaltic movement changes to a mature sequential beating pattern during heart looping stages. In zebrafish the primary cardiac pacemaker cells are located at the sinoatrial junction at the posterior pole of the heart. They can be distinguished from the surrounding myocardial cells by specific expression of the transcription factor Islet-1. Thus far, little is known about the molecular pathways and factors underlying pacemaker cell development. Limited by the low number of Isl1 positive pacemaker cells, large-scale gene expression analysis has been challenging.
Here we have taken advantage of *tomo*-seq as an unbiased approach to gain transcriptome-wide expression data in a highly spatially restricted manner (Junker et al., 2014; Kruse et al., 2016). Embryonic hearts were dissected from 2 days old embryos and embedded in tissue freezing medium for cryo-sectioning. Total RNA was isolated from each individual 10µm section and processed for RNA-seq.
We have obtained genome-wide transcription information allowing for the spatial dissection of expression profiles in the developing heart tube. Regions of specific gene expression within the heart allow the identification of novel genes involved in the pattering processes of the heart. We could localize the pacemaker cells within the tissue sample and analyze the total transcriptome of the pacemaker domain. Initial gene ontology analysis showed enrichment in migratory, progenitor state cells.
Genes highly expressed in the pacemaker domain were analyzed with regard to their possible role in pacemaker cells development. Specific expression of several components of the wnt-signaling pathway indicated a novel role for the wnt pathway in the patterning and development of the pacemaker cells. Experiments using a Wnt reporter indicated that Wnt signaling acts downstream of Isl1 activity. Furthermore, cardiomyocyte-specific inhibition of canonical wnt signaling during specific stages of heart morphogenesis affected the heart rate, suggesting an important role for Wnt signaling in pacemaker function.

Lineage analysis of ventricular trabeculae to decipher the role of Nkx2-5 in conduction system development

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During cardiac development, transient invaginations of the myocardium, termed trabeculae, appear at the endocardial surface of the ventricles. Trabecular compaction during fetal development is an essential step in generating a functionally competent ventricular wall. Trabeculae also contain progenitor cells of the ventricular conduction system (VCS), a complex network of Purkinje fibers that controls the rapid propagation of electrical activity in the ventricles. Defects in ventricular compaction and conduction have been observed in patients and mutant mice carrying mutations in *NKX2-5*, encoding a key transcriptional regulator of heart development. In order to analyze the link between trabecular fate and VCS differentiation, we carried out a genetic lineage analysis of trabecular fate using *Cx40-CreERT2* mice expressing a tamoxifen-inducible Cre recombinase in ventricular trabeculae and later in the definitive ventricular conduction system. These mice were crossed with *Rosa26-Confetti* mice for a prospective clonal analysis of trabeculae. Our results show that the peripheral VCS segregates progressively during embryonic development. Cells exclusively fated to give rise to the VCS are present in the trabecular compartment as early as E9.5. Specification of new conductive myocytes within the trabecular compartment during subsequent development contributes to the formation of a complex Purkinje fiber network at birth. We performed the same lineage tracing experiments in *Nkx2-5* heterozygous mice with severe hypoplasia of the Purkinje fiber network at adult stages. The number of Purkinje fibers originating from early *Nkx2-5* hapolinsufficient progenitors is identical to control. In contrast, we observed a progressive increase of trabecular cells that do not give rise to the VCS at later stage of development. This suggests that Nkx2-5 plays a role in the progressive recruitment of trabecular cells into the VCS as well as later in maintenance of the conductive phenotype. Overall this study highlights the early segregation of the ventricular conduction system lineage at the onset of trabeculation and the role of Nkx2-5 at later stages of the formation of the Purkinje fiber network.

Heart regeneration in the Mexican cavefish

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Complete regeneration of the adult heart after injury is a feature exclusive to a limited number of species, such as the zebrafish and salamander. Injury of the ventricle of a zebrafish heart results in the wound tissue being replaced by new, functional cardiac muscle. In contrast, in human patients fortunate enough to survive myocardial infarction, the dead heart muscle is substituted by a permanent fibrotic scar that will never be replaced by new heart muscle, and may cause severe contractile dysfunction, resulting in heart failure and even recurring myocardial infarction. Whilst the zebrafish has proven to be a useful model to study human diseases, inter-species differences have confounded direct comparisons between mammalian (rodents, human) and fish. Genome-wide data sets, and in particular the promise of comparative transcriptomics, has thus far failed to deliver the key factors that might distinguish between tissue regeneration and scar-based wound healing. Here, we present a new model to overcome the caveats of interspecies variation in cardiac wound healing and regeneration: the Mexican cavefish. *Astyanax mexicanus* is a single fish species comprising cave-dwelling and surface populations that uniquely allows comparison of an adult regenerative and scarring response within one species. While *Astyanax mexicanus* surface fish regenerate their heart after injury, their cave-dwelling counterparts cannot and form a permanent fibrotic scar, similar to the human heart. This lack of heart regeneration is neither linked to fin regeneration nor the cavefish specific eyeless and albino phenotype. Myocardial proliferation peaks at similar levels in both surface fish and Pachón one week after injury. However, in Pachón this peak coincides with a strong scarring and immune response and, ultimately, cavefish cardiomyocytes fail to replace the scar. Our study provides evidence that, unlike findings in zebrafish, myocardial turnover is not the sole driver of heart regeneration and that successful heart regeneration entails a delicate interplay between myocardial proliferation and scarring.

A new *ex vivo* model to study cardiac fibrosis

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Background/Introduction: Fibrosis is a characteristic of several cardiac diseases that lead to heart failure. No effective treatment exists, therefore the urgent need is present to obtain new insights in this disease in order to develop better therapeutics that will aid in its prognosis and treatment. *In vivo* mouse models (aortic banding, coronary artery ligation) have offered great insights in the definition and characterization of this disease. However, controlled manipulation of single or specific combination of potential effectors cannot be achieved.

Purpose: The goal of our study is to develop an *ex vivo* culture system in which cardiac fibrosis can be induced and regulated in the adult mouse heart.

Methods: Mouse hearts are isolated and cultured in the Miniature Tissue Culture System. In this ex vivo flow system a closed circulatory system is created in which the heart is perfused with medium for up to 2 weeks. After culture, the hearts are processed for histopathological analysis. Myofibroblast accumulation and collagen production are measured as indicatives of the fibrotic load.

Results: Cardiac fibrosis can be induced within a week throughout the ventricular myocardium as shown by the high number of collagen-expressing myofibroblasts (alpha smooth muscle actin-positive cells). After 2 weeks, the extracellular matrix accumulation is evident by the high collagen deposition in the ventricular myocardium. The fibrosis induced in the *ex vivo* cultured heart is age- and flow speed-dependent. By selective inhibition of the receptor of the profibrotic growth factor TGFb pathway, a strong reduction of the fibrotic load was observed.

Conclusions: We have developed an *ex vivo* flow system, which allows induction of cardiac fibrosis *ex vivo* in intact adult mouse hearts. In this powerful tool mechanical or biochemical stimuli can be altered, individually or in combination, in order to model the different stages of cardiac fibrosis. Moreover, the use of genetically modified mice hearts will give the opportunity to understand the cellular and molecular mechanisms underlying fibroblast expansion and function, revealing new therapeutic targets, paving the road towards the treatment of cardiac fibrosis.

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Unraveling the underlying embryological origin of cardiomyopathy

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A quarter of deaths in the UK are due to heart disease and there is increasing evidence that abnormalities occurring *in utero* can predispose to disease in later life. Hypertrophic cardiomyopathy (HCM) commonly presents during adulthood and disease-causing mutations have been reported in the sarcomeric genes. HCM has a variable degree of phenotypic heterogeneity, even within the same family. Therefore, this variability may be influenced by the presence of additional genetic modifiers.
The small GTPase, ROCK, is a serine threonine kinase, which is involved in a wide range of functions including regulation of the cytoskeleton, cell migration and cell polarity. Importantly, ROCK contributes to the regulation of actin-myosin contraction in cardiomyocytes. We are studying the role of ROCK within the developing cardiomyocytes using transgenic mouse models.
Conditional downregulation of ROCK in the cardiomyocytes from embryonic day (E)9.5 in transgenic mice altered the development of the ventricular wall, as the myocardium was thinner with longer noncompacted trabeculae evident in the mutant embryos. Despite this, these mice survived to adulthood, displaying many symptoms consistent with HCM. A detailed intracellular analysis of the cardiomyocytes has revealed that from E10.5 there is disruption of troponin expression and the sarcomeres are abnormal, which then causes the cells to undergo temporary cell cycle arrest and subsequent compensatory hypertrophy. Disruption of the sarcomeres also affects the contractility of the hearts. We believe that ROCK specifically phosphorylates Troponin I and that this is required for the normal assembly and function of the sarcomeres, and when this process is disrupted this predisposes to HCM in adulthood. Therefore, we propose that ROCK may be a novel genetic modifier in the progression of HCM.

Transdifferentiation of cardiomyocytes during heart regeneration in the adult zebrafish

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During heart development, mesodermal progenitors from the first heart field (FHF) form a primitive cardiac tube to which cells from the second heart field (SHF) are added. The role of SHF and FHF-derived cells during regeneration has not been determined. The zebrafish is an established model organism to study heart regeneration, in which pre-existing cardiomyocytes proliferate to replace the lost myocardium. FHF and SHF progenitors have also been described in this species, but the extent of their contribution to the adult heart is unknown. We found that the ventricular myocardium in the adult zebrafish was mainly derived from FHF progenitors, with a small contribution from SHF progenitors. Notably, we found that ablation of ventricular FHF-derived cardiomyocytes in the embryo is compensated by expansion of SHF-derived cells. If this observed cardiomyocyte plasticity persist in the adult is unclear. While clonal analysis suggested that cortical myocardium is rebuilt by cardiomyocytes from the same layer, we describe here that trabecular cardiomyocytes can transdifferentiate into cortical myocardium during adult heart regeneration. The described versatility of rebuilding a vertebrate heart in a model organism shall bring us closer to design therapies for promoting myocardial regeneration in mammals.

Single-cell mRNA-sequencing of the regenerating zebrafish heart reveals distinct populations of cardiomyocytes.

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In contrast to adult mammals, zebrafish can completely regenerate heart injuries by proliferation of pre-existing cardiomyocytes. However, the signaling pathways and molecular mechanisms underlying these processes remain largely unknown. Recent advances in technology allow us to obtain whole-genome transcriptomics data from tissue sections and even single cells.

We have demonstrated that *tomo*-seq generates whole transcriptomics data with spatial resolution across one axis in a tissue of interest (Junker et al., 2014, Cell; Kruse et al., 2016, Methods Cell Biol). The drawback of this technique is that it does not provide cell-type specificity. Single-cell sequencing, on the other hand, offers whole transcriptome information of single cells, with the limitation that cells cannot be traced back to their original location in the tissue. We hypothesize that the combination of data from these two techniques will yield to the identification of novel cardiomyocyte-specific genes involved in heart regeneration in the zebrafish. Here, for the first time, we report single-cell mRNA sequencing of freshly isolated cardiomyocytes from the regenerating fish heart.

Tomo-seq analysis on cryo-injured zebrafish heart revealed upregulation of *nppa* and *nppb* expression in the myocardial border zone, a region enriched with proliferating cardiomyocytes. We generated a transgenic Tg(*nppaBAC::YFP*) line and which recapitulates endogenous *nppa* expression. In addition our results show a high correlation between *nppa/YFP* expression and PCNA, a marker for proliferating cells, validating that this line could be used to enrich for proliferating cardiomyocytes. Cells were isolated from injured hearts at 7 days post injury, sorted for *YFP*-expression by flow cytometry and sequenced. Using bioinformatics a variety of cell types were identified. Most interestingly, distinct populations of cardiomyocytes were found with different states of differentiation - ranging from immature cardiomyocytes to fully differentiated heart muscle cells. In addition, analysis of the single-cell RNA-seq data revealed distinct cellular processes that are activated in the immature and proliferating cardiomyocytes.

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Poster abstracts

1. The roles of the Peroxisome Proliferator-Activated Receptor β/δ (PPAR β/δ) during cardiovascular system development in the chick embryo.

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Many decades of genetic selection have yielded outstanding performing broilers which are reared for meat. The broilers were selected for agricultural-preferred properties; however, cardiac function was not one of them. This selection also contributed to a substantial allometric imbalance in which the fast growing chick is supported by a comparatively small heart. Thus, broilers often suffer from poor cardiac function which leads to health deterioration, poor performance and premature death. We hypothesize that improving cardiovascular formation and function during early stages of embryogenesis may have a critical role in chick development, posthatch. Importantly, bettering the embryonic cardiac function will contribute to the animal welfare at adulthood, as well as to improved broilers performance. The family of the nuclear receptor Peroxisome Proliferator-Activated Receptor (PPAR) has three members, α, β/δ and γ, and we show that in the chick embryo, PPAR β/δ is the only member expressed in the heart at the time of the very first heart beats. The PPAR β/δ pathway is known to be involved in metabolic balance and cardiac endurance during adulthood. Thus, it is a direct target for treatment approaches in diabetes, hypertension and cardiac dysfunction. However, the roles of the PPAR β/δ pathway during early stages of embryogenesis are poorly understood. We describe the expression pattern of PPAR β/δ during embryogenesis and manipulate the activity of the pathway by administrating available agonists and antagonist molecules which were specifically designed as drugs for humans. We show that the PPAR β/δ pathway is involved in angiogenesis, cardiogenesis and overall growth rate.
To better quantify the PPAR β/δ –related effects on cardiac function, we developed an in-ovo live imaging tool that allows for cardiac output measurement. This, combined with state-of-the-art 3D modeling techniques, which include high resolution micro-CT and the cutting-edge optical High Resolution Episcopic Microscopy (HREM) system, allows us to accurately characterize the structure and function of the early embryonic heart at a resolution never attained before.

2. Lineage tracing reveals the cellular origin of the adventitia of the ascending aorta

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Pathology of the arterial wall is one of the leading causes of death in the UK. The arterial wall has three layers, an endothelial lining, a thick layer of smooth muscle and an outer fibrous adventitial layer that contains a range of different cell types including fibroblasts, blood vessels, lymphatic vessels and neurons. Previous studies have suggested that the endothelial layer of the ascending aorta is derived from the second heart field (SHF), whereas the smooth muscle (media) has spatially-restricted origins from both the SHF and from the neural crest. In order to gain a better understanding of how the adventitia develops and matures, we asked whether the cells within the adventitia of the ascending aorta and aortic arch share an embryonic lineage with the smooth muscle cells of the media, and whether these cells differentiate to form all of the cell types found in the adult adventitia.
Using Cre-lox driven lineage tracing in adult mice, we show that SHF cells, identified using *Mef2c Cre*, form a subset of fibroblasts within the adventitia of the ascending aorta, but not those of the aortic arch or descending aorta. Neural crest cells, labelled by *Wnt1 Cre*, gave rise to a subset of fibroblasts within the adventitia of the aortic arch, with only a few *Wnt1 Cre*-derived neurons found within the ascending and descending aorta. Other cell types, including blood and lymphatic vessels, were largely unlabelled by *Mef2c Cre* or *Wnt1 Cre* and therefore their origins were unclear. Thus, the origin of the fibroblasts within the adult adventitia mirrored that of the media. The origin of the adventitial fibroblasts also showed the same boundaries as the media, with distinct boundaries apparent in the ascending aorta and aortic arch reflecting their origins from the aortic sac and pharyngeal arch arteries, respectively. Hence the recruitment of cells from the local environment likely contributes to the complex nature of the adult adventitia.

3. Gross-morphological aspects of the adult zebrafish heart: bilateral asymmetry

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Specification of the left-right body axis plays an important role in development of vertebrate hearts. The heart is the first organ to acquire a bilateral asymmetric shape during embryonic development and heart asymmetry is a prerequisite for correct function of the circulation of lung-breathing vertebrates. Asymmetric morphogenesis of the embryonic vertebrate heart is a phylogenetically highly conserved process. Therefore, the zebrafish has become a well-establish model organism to study cardiac ontogenesis. Recent data show that the atrium and ventricle of the developing zebrafish heart become repositioned to the body midline at early larval stages (5dpf.). Furthermore, it is frequently stated in the literature that adult teleost hearts are bilaterally symmetric structures. This suggests that, in contrast to other vertebrates, cardiac asymmetry may be a transitory phenomenon in teleosts.

Gross anatomy of adult zebrafish hearts (WT, 1-year-old) was studied on 45 specimens by micro-dissection and SEM.

The ventricle of adult zebrafish hearts has the shape of a three-sided (dorsal, right-ventral, left-ventral) pyramid. Its base is connected to the bulbus arteriosus, which lies in the body midline. Its apex points to the ventral midline at the caudal end of the pericardial cavity. The right-ventral surface of the ventricle contacts the right pericardial wall. On the left side, the left atrial appendage is interposed between the pericardial wall and the left-ventral surface of the ventricle. The atrium lies dorsal and to the left of the ventricle.

The adult zebrafish heart is a bilaterally asymmetric structure. Its anatomy reflects the asymmetry of the embryonic heart.

4. Embryonic origins of the great arteries

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Background: Developmental abnormalities of the proximal thoracic aorta are a common accompaniment to congenital heart malformation. In adulthood, specific areas are affected by cardiovascular disease processes which have been related to physical forces. However, it has also been suggested that the distribution of different cell lineages in the vessel wall may focus pathologies. Avian and murine studies show that the tunica media in the root of the great arteries is derived from the second heart field (SHF) whereas neural crest cells (NCCs) give rise to the tunica media in the aortic arch.

Aim: We sought to establish the origins of the tunica intima and tunica adventitia and to determine whether lineage boundaries of the great arteries persist into adulthood. In addition, we asked if SMC cytoskeletal phenotype was linked to these origins.

Methods: R26R eYFP$^{F/F}$ transgenic reporter mice were crossed with mice carrying transgenes for Mef2c-AHF-Cre and Wnt1-Cre to track cells derived from the SHF and NC respectively. The eYFP epitope was detected by immunohistochemistry on paraformaldehyde fixed paraffin embedded tissue sections.

Results and Conclusions: The majority of smooth muscle cells (SMC) in the root of the aorta and pulmonary artery trunk are derived from the SHF. The outer media and adventitia of the ascending aorta and pulmonary artery trunk are also formed from the SHF whereas the inner media of the ascending aorta and pulmonary artery trunk, as well as the media and adventitia of the aortic arch, are derived from the NCCs. In contrast, the intima of the pulmonary artery trunk, ascending aorta and aortic arch is derived solely from the SHF. These distinct boundaries are maintained as the vessels mature.

The SMC cytoskeletal proteins αSMA and SM22α are expressed at high levels throughout the media of the great arteries; except for regions in the proximal right subclavian artery and in the aortic arch, between the left common carotid and left subclavian arteries. These actin deficient stripes correspond to the regions of the mature aortic tree that are derived from the embryonic fourth pharyngeal arch arteries and share their distal boundary with the NC lineage boundary in the media. Desmin was localised most strongly to the outer media of the ascending aorta and pulmonary trunk, generally in cells from the SHF. Desmin was also strongly expressed in the NC derived ductal ligament.
These studies indicate that cell lineage boundaries differ in the intima, media and adventitia, revealing variation in their embryonic origin throughout the aorta and pulmonary artery. SMC proteins show differential expression patterns that do not map exclusively to lineage boundaries but instead appear to reflect the developmental structure they are derived from. Future studies will elucidate whether the differences in embryonic origin throughout the great arteries influence the development and progression of vessel pathology.

5. Investigating heart size differences during the early development of *Astyanax mexicanus*

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Heart size regulation is one of the least understood processes in vertebrate developmental biology, even though disruption of such processes could lead to highly debilitating or lethal pathologies. The Mexican tetra (*Astyanax mexicanus*) exists as a river-dwelling (epigean) morph and cave-dwelling one (troglomorph), the latter of which appears to have a smaller ventricle size than the former at the adult stage. However, when this size difference arises during heart development and how atrium sizes change in the 2 morphs are unknown. In this project, the expression patterns of 4 genes (Myh6, Myl7, Myh7l, Nkx2.5) involved in heart development were examined (by *in situ* hybridisation) to investigate when and how heart size differences arise. The results of this project are the first to show that both the atrium and ventricle are smaller in the cavefish by 2dpf. A smaller atrium is also detectable in the cavefish at the 24h-stage. Together, the results suggest that the developmental mechanisms that contribute to a smaller heart size occur early in embryogenesis, possibly by a reduction in cell number. The identification of heart size differences in *A.mexicanus* could gain more insight into how is controlled during cardiac development, with potential relevance to cardiac pathologies in humans.

6. An organized thrombus with calcification in the left atrium is not rare and can cause decreased cerebral blood flow in elderly patients as assessed using brain perfusion SPECT

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[Background] Stroke is an important clinical problem that causes death or work disability; therefore, its prevention is crucial. An organized thrombus in the left atrium has the potential to cause ischaemic stroke and is considered rare. This study showed that organized thrombi are common in elderly patients and that they affect the cerebral blood flow. [Method] Thirty-four patients with pulmonary vein thrombi (PVT) were examined via a transesophageal echocardiogram (TEE) to assess a cardiac thrombus. The patients had hypertension, dyslipidaemia, diabetes mellitus, and palpitations. [Results] Twenty patients (58%) had an organized thrombus with calcification in the left atrium (Calcification group; 68 ± 9 years, 8 males, 12 females), and 14 patients did not have calcification (No-Calcification group; 73 ± 13 years, 8 males, 6 females). The average values of brain perfusion single-photon emission computed tomography (SPECT) were 1.27 ± 0.37 (Calcification) and 1.18 ± 0.35 (No-Calcification), indicating that the Calcification group showed decreased cerebral blood flow. After 3 to 6 months of treatment with a novel oral anti-coagulant (NOAC), the values of 20 patients (100%) in the Calcification group increased to 1.41 ±0.38 (decreased blood flow); however, the values of 12 (86%) patients in the No-Calcification group decreased to 1.13 ±0.40 (increased blood flow), indicating that NOACs increase cerebral blood flow and that calcified particles released from the resolving organized thrombus with calcification may decrease cerebral blood flow by occlusion of cerebral microvessels. [Discussion] An organized thrombus with or without calcification in the left atrium is considered rare; however, we found that this condition is common. An organized thrombus with calcification in the left atrium can cause cerebral microvessel occlusion. NOACs may prevent ischaemic stroke, dementia, Alzheimer's disease and cardiac syndrome X by increasing the microvessel blood flow, if treatment occurs before the formation of calcification. Research on genetic factors associated with calcification may lead to new diagnostic methods and treatments to prevent stroke.

7. Acetylation of TBX5 by KAT2B and KAT2A regulates heart and limb development

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Congenital Heart Defects (CHD) affect around 1 in 150 newborns and represent the most common form of congenital abnormality. The heart is the first organ to form and multiple different types of abnormality can arise during the course of development: from small holes in the walls of the cardiac chambers, to more complex defects which can eventually lead to an early death. Human genetic studies have shown that most of the faulty genes responsible for CHDs are transcription factors that regulate and control a wide range of events taking place during heart development, such as atrial and ventricular septation or great vessel formation. Early work from our lab led to the identification of mutations in TBX5, a T-box transcription factor, as the cause of Holt-Oram syndrome, a rare genetic condition that affects the development of the heart and upper limb. Further studies have confirmed TBX5 as a master regulator of cardiac development; however, how TBX5 activity is regulated during cardiogenesis remains largely unknown.
In this study, we report that histone acetyltransferases KAT2Aand KAT2B regulate TBX5 transcriptional activity. Using interactional and transcriptional assays, we show that both KAT2A and KAT2B physically interact with TBX5 *in vitro* and acetylate it at Lys339. Acetylation augments its transcriptional activity and is required for its nuclear export. To examine the *in vivo* role of *kat2a* and *kat2b* we performed gene knock-down experiments in zebrafish at early stages of development and examined the phenotypic consequences. Morpholino-mediated knockdown of *kat2a* or *kat2b* produced both heart and fin phenotypes similar to that observed in TBX5 mutants, which includes pericardial oedema, incomplete heart looping , lack of blood flow into the heart and absence of functional fins. The phenotypes found in MO-injected embryos were also observed when we introduced mutations in the *kat2a* or *kat2b* genes using the CRISPR-Cas system.

This work provides the basis for further experiments to understand the role of TBX5 and its modulator proteins during heart development

8. *Ezh2* deletion rescues the *Tbx1* haploinsufficiency phenotype

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Haploinsufficiency of the *Tbx1* gene in humans and mice is a cause of DiGeorge syndrome, a multiple congenital anomaly syndrome that includes also an important adult phenotype. The gene encodes a transcription factor of the T-box family, thus its canonical function is to regulate transcription by binding to regulatory genetic elements, and it can activate or repress transcription. Tbx1 interacts with chromatin components such as the SWISNF-like subunit Baf60a/Smarcd1 and histone methyltransferases. The current model for Tbx1 function is that it binds to its DNA sites where it recruits a SWI-SNF chromatin remodeling complex as well as histone modifying enzyme(s), setting the gene on a permissive state. In our effort to develop startegies to compensate for reduced dosage of Tbx1, and to understand transcriptional mechanisms of cardiac development, we tested the hypothesis that the Polycomb Repressor Complex 2 (PRC2) functions as an antagonist of the Tbx1-BAF-Mll3 complex.
To this end, we crossed $Tbx1^{Cre/+}$ (functionally heterozygous) and $Ezh2^{flox/+}$ mice and we found that the typical *Tbx1* haploinsufficiency phenotype, i.e. hypoplasia/aplasia of the 4th pharyngeal arch arteries (PAAs) was completely rescued in E10.5 $Tbx1^{Cre/+}$;$Ezh2^{flox/+}$ embryos (n=22, P=0.0006). Next, we tested whether pharmacological inhibition of the enzymatic activity of Ezh2 using GSK126 may also rescue the Tbx1+/- phenotype. Results showed no effect suggesting that rescue is not due to reduced enzymatic activity.
Next, we set to understand possible rescue mechanisms. We found that reduced dosage of *Ezh2* enhances the expression of *Tbx1* as well as that of target genes *Wnt5a* and *Gbx2*, in vivo and in P19CL6 cultured cells. Thus, Ezh2 is a negative regulator of *Tbx1*. Both *in vivo* (E9.5) and *in vitro* downregulation of Ezh2 results in a reduction of Ezh2 (and other PRC2 components) localization and H3K27me3 enrichment on three *Tbx1* regulatory sequences. We confirmed in tissue culture that the mere inhibition of the enzymatic activity of Ezh2 is insufficient to mimic these results. Thus, our results so far indicate that the PRC2/Ezh2 complex is a negative regulator of Tbx1 gene expression, and this may explain why heterozygous deletion of Ezh2 rescues the haploinsufficiency phenotype in *Tbx1+/-* embryos.

9. Msx1 heterozygosity modulates the Pax9-null cardiovascular phenotype.

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Development of the aortic arch arteries from the pharyngeal arch arteries is a complex process requiring the interaction of several tissue types and tightly regulated gene expression during embryogenesis. Using transgenic mice, imaging techniques and gene expression analysis we have investigated the role of the transcription factor *Pax9* in cardiovascular development. Pax9 is specifically expressed in the pharyngeal endoderm at mid-embryogenesis, and *Pax9*-null mice die at birth from defects in the formation and remodeling of the 3rd and 4th pharyngeal arch arteries resulting in absent common carotid arteries, aberrant right subclavian artery and interrupted aortic arch.

We wanted to identify genes that might be acting in the same genetic regulatory network as *Pax9* to control cardiovascular development. We selected the transcription factor Msx1 as this has been shown to interact with Pax9 in tooth development, and transgenic mice lacking *Msx1* and *Msx2* display cardiovascular defects. Surprisingly, we discovered that mice simultaneously null for *Pax9* and heterozygous for *Msx1* have a significantly reduced incidence of aortic arch defects compared to *Pax9*$^{-/-}$ mice. Although Pax9 and Msx1 proteins can interact during tooth formation, the expression of these two genes does not overlap in the pharyngeal arches, with Msx1 being expressed in neural crest cells. Immunohistochemistry labelling revealed that more neural crest cells were present within the 3rd pharyngeal arch of *Pax9*$^{-/-}$*;Msx1*$^{+/-}$ embryos compared to *Pax9*$^{-/-}$ embryos, which may suggest a role for these cells in protecting the 3rd pharyngeal arch artery from regressing as it does in *Pax9*$^{-/-}$ embryos. Our data therefore show that *Msx1* heterozygosity appears to modulate the *Pax9*-null cardiovascular phenotype by an as yet unrecognized mechanism.

10. miR-200b in cardiac development

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The transcriptional regulation of cardiovascular development requires precise spatiotemporal control of gene expression. A novel mechanism involving post-transcriptional regulation by small noncoding microRNAs (miRNAs) has recently emerged as a central regulator of distinct cardiogenic processes. The identification of miRNAs expressed in specific cardiac and vascular cell types has led to the discovery of important regulatory roles for these small RNAs during cardiomyocyte differentiation, cell cycle, conduction, and vessel formation. Here we show that *miR-200b*, which has been previously demonstrated to be significantly up-regulated during cardiomyocyte differentiation, modulates the contractile phenotype during *in vitro* embryonic stem cells (ESCs) cardiac differentiation. Interestingly, *miR-200b* regulates the expression of cardiac genes such as *Cx45* (connexin that form low conductance channel) and *Tbx5 (*a cardiac determinant gene). In this sense, RT-PCR analyses showed that *miR-200b* is expressed at E12.5 and E15.5 during heart development. Moreover, LNA *in situ* hybridisation experiments revealed that *miR-200b* is present in a cell subpopulation in the embryonic and fetal heart. We are currently characterizing the *miR-200b+* cardiac cell population by co-localization with specific cell-markers. Collectively, our data suggest that this *miRNA* might be a key molecule regulating cardiac cell lineage diversification during cardiac development.

11. Isl1 regulates temporospatial differentiation of the second heart field into cardiomyocyte, smooth muscle cell and fibrous lineages in the arterial roots

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Background: The arterial roots connect the ventricles to the ascending aorta and pulmonary trunk. They house the leaflets of the arterial (semilunar) valves, and in the case of the aorta, give origin to the coronary arteries. Abnormalities of the arterial roots are common and include aortic and pulmonary stenosis/atresia and bicuspid or dysplastic valve leaflets.
The outflow forms by addition of second heart field (SHF) cells to a primitive primary heart tube, which initially consists of only an inlet and outlet chamber. Previous studies have shown that the initial wave of SHF cells differentiates into cardiomyocytes and a second wave of SHF-derived cells then contributes smooth muscle cells (SMC) and fibroblasts to the arterial trunks. Neural crest cells (NCC) are also a source of SMC to the aorta and pulmonary trunk. The developmental mechanism by which the arterial roots form is unknown

Aim: We sought to identify the progenitor lineages that comprise the aortic roots and also understand the mechanism by which both myocardium and SMCs can be derived from the SHF.

Method: Using a combination of Cre-lox technology, immunohistochemistry and RT-PCR, we evaluated the formation of the aortic roots from embryonic day E9.5 to postnatal day P21.

Results and conclusion: These studies confirmed that SHF cells within the epithelial dorsal pericardial wall give rise to the cardiomyocytes of the proximal outflow vessels and that a later wave of these cells forms the adventitia that coats the ascending aorta and pulmonary trunk. In contrast, it is the mesenchymal component of the SHF, together with NCC, that contributes the SMC that form the media of the ascending great arteries. Thus the precursors of cardiomyocytes, SMC and fibroblasts are temporally and spatially distinct in the SHF.
We show that nuclear or cytoplasmic expression of Isl1 is associated with different differentiation pathways to either smooth muscle or myocardial fate.

12. Identification of novel downstream targets of the GATA transcription factors during cardiomyogenesis

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The heart is the first organ to form in the vertebrate developing embryo, yet the individual roles of transcription factors (TFs) and signalling molecules involved in regulating heart development are not fully understood. Of noticeable importance, the GATA family of TFs (GATA4-6) have been identified as crucial, evolutionarily conserved pro-cardiogenic factors, as their genetic ablation in the developing frog and mouse embryo causes significant heart defects. However, how the GATA TFs regulate heart development and particularly what transcriptional targets they regulate is not well characterised. Recent RNA-Seq analysis in our research group following knockdown of the GATA TFs in *Xenopus laevis* have identified a number of putative uncharacterised downstream targets. To determine whether a conserved role of the identified targets exists during mammalian cardiomyogenesis, genetic manipulation utilising loss-of-function as well as overexpression will be performed in mouse embryonic stem (ES) cells. Such experiments will establish whether the mouse homologs are also regulated by GATA TFs and investigate the role of these novel downstream GATA targets in cardiomyogenesis. This will be coupled with investigating the role of Wnt signalling, as both canonical and non-canonical pathways have been demonstrated to functionally integrate with GATA TFs, and are essential for heart muscle development.

13. Quantification and developmental expression of alternatively spliced transcripts from duplicated zebrafish mapk8 (jnk1) genes

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Background: Congenital heart defects (CHD) are seen in almost one per cent of live born infants, and a frequent finding in first trimester miscarriage. Mice carrying mutations for proximal members of the non-canonical Wnt, planar cell polarity pathway exhibit similar cardiac defects such as double outlet right ventricle. Little is known about the requirement for one specific distal mediator of this pathway, c-Jun amino-terminal kinase (Jnk). The Mapks are stress responsive kinases, but are also known to act as major regulators of cytoskeleton, transcription and apoptosis. Mouse mutants for *Mapk8*, *9* and *10* (Jnk 1, 2 and 3) show no overt phenotype, suggesting significant compensation from other jnk family members. However, although double mutants for *Mapk8/9* present severe gastrulation defects and are embryonic lethal, *Mapk8/10* mutants have an abnormal cardiac phenotype, before dying at E11. Zebrafish have undergone a genome duplication event and there are two jnk1 genes - *jnk1a* on chromosome 13 and *jnk1b* on chromosome 12. Transcripts from these *jnk1* genes undergo two splicing events: alternative inclusion of either exon 7 or 8 and an alternative intron-exon splice site to produce a long or short 3' end.

Aim: We set out to map the gene expression patterns of all four alternatively spliced transcripts from both genes. We asked if there might be tissue or developmental stage specificity for particular transcripts and splicing events of relevance for heart development.

Method: We designed an RT-PCR assay based on both RT-PCR and restriction digest to quantify the relative proportions of all eight genes products throughout development and designed whole mount *in-situ* probes to provide spatial detail.

Results and Conclusion: Bioinformatic analysis suggested that there was strong conservation of transcript sequence between human and zebrafish. However, it appeared that some transcripts from each zebrafish *mapk8* gene more closely resembled the human gene products than the other. RT-PCR and restriction digest analysis indicated that there were progressive changes in the transcript pool throughout development and also that these favoured the more human-like transcripts. Further evidence of tissue specific expression of the zebrafish *mapk8* genes was obtained by whole mount in-situ hybridisation analysis.

14. Investigation of the role of Tbx1 in the posterior second heart field.

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The Second Heart Field (SHF) is a population of cardiac progenitor cells in pharyngeal mesoderm that contributes to myocardium at the poles of the elongating heart tube. Perturbation of SHF development results in a spectrum of common forms of congenital heart defects, exemplified by 22q11.2 deletion or DiGeorge syndrome (22q11.2DS). Distinct anterior and posterior SHF subpopulations have been identified contributing to right ventricular and outflow tract myocardium at the arterial pole and atrial and atrial septal myocardium at the venous pole, respectively. However DiI, genetic lineage and clonal analysis experiments have shown that common progenitor cells contribute to both poles of the heart tube and the mechanisms regulating emergence of distinct arterial and venous pole progenitor populations are poorly understood. Tbx1, encoding a T-box transcription factor, is the major 22q11.2DS candidate gene and regulates proliferation and differentiation in the SHF, being required for the addition of Hoxb1 expressing progenitor cells to the outflow tract. Tbx1 also regulates venous pole progenitor cell contributions to the dorsal mesenchymal protrusion, resulting in atrioventricular septal defects in Tbx1 null hearts. Here, using genetic lineage tracing and immunofluorescence labeling we demonstrate that a Tbx5 positive transcriptional domain emerges in Tbx1, Fgf10 and Mef2c-AHF-Cre expressing cells in the posterior SHF at the 4 somite stage. Analysis of Raldh2 expression and function and embryo culture with retinoic acid antagonists suggest that Tbx5, a T-box transcription factor implicated in Holt-Oram syndrome, is activated in this domain in response to retinoic acid signaling. Subsequently, in a Tbx1-dependent process, Tbx1, Fgf10 and Mef2c-AHF-Cre expression is downregulated in the posterior SHF and becomes restricted to arterial pole progenitor cells in the anterior SHF, resulting in the formation of a sharp transcriptional boundary between these domains. Electroporation and embryo culture experiments suggest that negative regulation of Tbx1 by Tbx5 also contributes to boundary formation. Our results provide new insights into progenitor cell patterning during second heart field deployment.

15. Vegfr3 overexpression partially rescues the brain vascular defects of *Tbx1* mutants

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The transcription factor TBX1 is the major gene involved in 22q11.2 deletion syndrome (22q11.2DS). Using mouse models of these diseases, we have recently shown that TBX1 regulates the *VEGFR3* and *DLL4* genes in brain endothelial cells (ECs) and that this interaction is critical for the development of the brain vasculature (Cioffi 2014). In particular, loss of *Tbx1* in mice causes global brain vascular defects, comprising vessel hyperplasia, enhanced angiogenic sprouting and vessel network disorganization. Brain vessels of *Tbx1* mutants are also functionally impaired. It has been shown that endothelial deletion of *Vegfr3 in vivo* caused brain and retinal hypervascularization by inhibition of Notch signaling (Tammela 2011). This s result was surprising because previous data showed *Vegfr3* as a pro-angiogenic factor.

We are using in vivo and cell-based approaches to study the interplay between Tbx1 and Vegfr3 in brain vascularization. First we manipulated VR3 expression in cultured endothelial cells and then we performed a functional assay (matrigel) based on the ability of the cells to form microtubules. We transfected HUVECs with a transgene carrying the full length murine *Vegfr3* cDNA (TgVegfr3) and we found a decreased number of the branchpoints; then we silenced *Vegfr3* by RNAi and we observed increased microtubule branching. Interestingly, the co-transfection of siRNA*TBX1* and TgVegfr3 partially rescued the hyperbranching phenotype.

We evaluated brain vasculature in mutant embryos by immunohistochemistry with a specific marker of the brain microvasculature. Our results showed that Vegfr3 overexpression partially rescues brain vascular hyperplasia both in *Tbx1*$^{Cre+}$ embryos (rescue of about 88%) both in *Tbx1* null embryos (rescue of about 59%).

Our data suggest that *Vegfr3* is a major effector of Tbx1 function in ECs, and the modulation of its expression is able to rescue the hyperbranching phenotype in EC-derived defects caused by the loss of *Tbx1* in vivo and in cultured ECs. In order to gain insights into possible mechanisms that may determine the in vivo and invitro rescue we are testing for a genetic interaction between *Tbx1* and *Vegfr3* in brain.

16. Dissecting the functional role of microRNAs in the chicken proepicardium

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The proepicardium is located with the septum transversum at the venous pole of the heart. It is composed by a small cell cluster, around 200 cells, that detaches and subsequently anchors into the surrounding ventricular chambers, contributing to distinct cardiac cell types, i.e. smooth muscle vascular cells, coronary endothelial cells, cardiac fibroblasts as well as to the outmost cardiac layer, the epicardium. Furthermore, besides the demonstrated cell plasticity of the embryonic proepicardium *in vivo*, *in vitro* proepìcardial cells can differentiate into cardiomyocytes. Classically the adult epicardium has been considered merely as a structural layer, however recent evidences demonstrate that contribution of the epicardium in fundamental in distinct experimental models of cardiac injury, providing pivotal cues for cardiac regeneration.

MicroRNAs are small non coding RNAs, 18-25 nucleotides, which are capable exerting a tight mRNA post-transcriptional regulation by either promoting transcript degradation or blocking protein translation. A fundamental functional role for microRNAs in epicardial development was provided in epicardial deficient *Dicer* mice, whose display embryonic lethality with abnormal vascular development.

In this study we aim to determinate candidate microRNAs that can selectively direct proepicardial cells to differentiation cell lineages that can serve as regenerative therapeutic tools. For this purpose, proepicardial explants were cultured in vitro, transfected with distinct microRNAs and markers for EMT, cardiogenesis, vasculogenesis and fibrogenesis were screened by qPCR. We observed that multiple microRNAs can positive influence EMT progression (e.g. miR-27, miR-21, miR-100, mir-125) at the expenses of cardiomyogenic differentiation, whereas others, such as miR-195 and miR-223, respectively, increase cardiomyogenic differentiation. Our results provide an entry point to use these molecules as therapeutic tools in the injured heart.

17. Human fetal and adult epicardial derived cells: a novel model to study their activation

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Background: The epicardium, a cell layer covering the heart, plays an important role during cardiogenesis providing cardiovascular cell types and instructive signals, but becomes quiescent during adulthood. Upon cardiac injury the epicardium is activated, which includes induction of a developmental gene program, epithelial-to-mesenchymal transition (EMT) and migration. However, the response of the adult epicardium is suboptimal compared to the active contribution of the fetal epicardium to heart development. To understand the therapeutic value of epicardial derived cells (EPDCs), a direct comparison of fetal and adult sources is paramount. Such analysis has been hampered by the lack of appropriate culture systems.

Methods: Human fetal and adult EPDCs were isolated from cardiac specimens obtained after informed consent. EPDCs were cultured in the presence of an inhibitor of the TGFβ receptor ALK5. EMT was induced by stimulation with 1ng/ml TGFβ. PCR, immunofluorescent staining, scratch assay, tube formation assay and RT2-PCR for human EMT genes were performed to functionally characterize and compare fetal and adult EPDCs.

Results: In this study, a novel protocol is presented that allows efficient isolation of human EPDCs from fetal and adult heart tissue. In vitro, EPDCs maintain epithelial characteristics and undergo EMT upon TGFβ stimulation. Although similar in several aspects, we observed important differences between fetal and adult EPDCs. Fetal and adult cells display equal migration abilities in their epithelial state. However, while TGFβ stimulation enhanced adult EPDC migration, it resulted in a reduced migration in fetal EPDCs. Matrigel-assays revealed the ability of adult EPDCs to form tube-like structures, which was absent in fetal cells. Furthermore, we observed that fetal cells progress through EMT faster and undergo spontaneous EMT when TGFβ signaling is not suppressed, indicating that fetal EPDCs more rapidly respond to environmental changes.

Conclusion: Our data suggest that fetal and adult EPDCs are in a different state of activation and that their phenotypic plasticity is determined by this activation state. This culture system allows us to establish the cues that determine epicardial activation, behavior, and plasticity and thereby optimize the adult response post-injury.

18. COUP-TFII plays distinct tissue-dependent roles during cardiac chamber morphogenesis

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COUP-TFII is an orphan nuclear receptor which is expressed by multiple embryonic tissues. During heart chamber morphogenesis, different COUP-TFII expression levels have been recorded in the atrial myocardium and endocardium as well as in the epicardium. COUP-TFII-null mice die around E9.5 from severe cardiovascular defects. COUP-TFII specific deletion in the myocardium and endocardium identified a unique transcriptional role for this molecule in the development of the atrial chambers and atrioventricular (AV) valves, respectively. On the other hand, COUP-TFII deletion using a partial epicardial driver (Gata5c-Cre) suggests a role for COUP-TFII in epicardial and coronary vasculature development. Using a robust proepicardial/septum transversum mouse Cre line (G2-Gata4-Cre), we show that COUP-TFII deletion in this cardiac inflow region, containing various cardiovascular progenitor lineages, affects 1) second heart field (SHF) cells fated to incorporate to the venous pole of the heart, and 2) epicardial progenitor cells at the proepicardium. Cardiac inflow defects affect atrial septation and can associate with AV defects. Interestingly, proepicardial cells normally contribute to epicardial formation, but both compact ventricular myocardial growth and prospective coronary artery formation are disrupted. We suggest that COUP-TFII plays pleiotropic, tissue-dependent roles during cardiac embryonic development.

19. Altered haemodynamic load leads to defective atrioventricular valve formation

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Intracardiac haemodynamics is crucial for normal cardiogenesis, with recent evidences showing valvulogenesis is haemodynamically dependent and is inextricably linked with shear stress [1, 2]. Valve anomalies have previously been found to be associated with genetic mutations, and a recent study showed that the OFT valve development was affected upon alteration of haemodynamics [3-5]. However, how the abnormal haemodynamics impacts the atrioventricular valve (AV) development is still poorly understood. OFT-banding was performed on HH21 hearts (chamber septation in progress, endocardial cushions initiated) with harvesting at HH26 (atrial septation complete, fusion of superior and inferior cushions), HH29 (fused cushions mature, primordial AV elongating and heart septated) and HH35 (primordial AV undergoing remodelling). Dysmorphic AV primordia were seen upon altered haemodynamics in histological analysis at HH29 (n=19/31) and HH35 (n=6/7) but not at HH26 (n=0/5). Stereological analysis also revealed that the primordial AV is smaller in the banded hearts at HH29 (n=7 OFT-banded hearts and n=12 controls; $p<0.002$). The deformed HH29 primordial AV was further characterized by a decrease in apoptosis, and the aberrant expression of extracellular matrix (ECM) characteristics of endocardial cushions alongside with the shear stress responsive genes by in situ hybridization and real time PCR. In addition, dysregulation of ECM proteins such as fibrillin-2, type III collagen and tenascin were further demonstrated in more mature primordial AV leaflets at HH35, with a concomitant decrease of ECM cross-linking enzyme, transglutaminase-2.

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20. Chicken enhancer assays; an alternative for mouse F0 screens

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Currently, the mouse represents the norm for the cardiac four chambered model 'in vivo' system when it comes to enhancer research. Possible alternatives for localizing heart enhancer expression are the zebrafish and lower eukaryotes such as Drosophila. In terms of their cardiac anatomy, both are quite distinct from higher vertebrates. In this respect, although perhaps as evolutionary distant as the Zebrafish, the chicken heart more closely resembles that of mammals and cardiac development proceeds much in the same way as that of the mammalian heart.
We have developed a lentiviral based enhancer assay that can be used to interrogate localized enhancer expression during chick development. Using several characterized enhancers we show that GFP expression can be driven in recognized localized expression patterns and that this pattern can be interrupted if enhancer components are mutated. Further, using different methods of culturing and combined with the fact that lentiviral derived DNA integrates into the host genome, different temporal expression patterns can be studied. Our current studies aim at making use of this cost effective system to study the effects of human common and rare variants on enhancer function.

21. Tropomyosin E62K mutation, but not E62Q, leads to cardiomyocyte hypertrophy

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A new mutation (g.1465 G>A, E62K) in the gene coding for human tropomyosin 1 (TPM1) has been identified in a patient diagnosed with Hypertrophic Cardiomyopathy at the Regional Hospital "Ciudad de Jaén". In this study we proposed that the change of glutamic acid for lysine at position 62 of the peptide sequence (E62K) could be dysfunctional, therefore could be causative of the disease. Since a mutation in the same gene position but affecting differently (E62Q) has previously been identified in a family with the same cardiac condition, we decided to study in parallel its putative functional defects. For this purpose, we have structured our experimental studies using site-directed mutagenesis technique by which we have selectively introduced the corresponding nucleotide changes (E62K and E62Q, respectively). Transient transfection assays using these plasmids into HL-1 cardiomyocytes allowed us to reveal the E62K mutation, but not E62Q, causes increased cell volume, a response which is further enhanced after treatment with pro-hypertrophy agents such as angiotensin II. Thus, our results show that E62Kmutation, but not E62Q, causes functional changes in the TPM1 protein.

22. Analysis of cardiac tissue samples from patients with congenital heart defects and non-failing heart samples shows distinct DNA methylation pattern in different heart chambers

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Aberrations of DNA methylation, an important epigenetic modification of the genome, have been associated to a growing number of human diseases, including congenital heart disease (CHD). Being essential during early mammalian development and regulating many cellular processes, DNA methylation plays a key role in tissue differentiation. Up to now, several tissue-specific differentially methylated loci have been shown in different organs, but only little is known about methylation patterns in the human heart. Therefore, we systematically analyzed tissue samples from different heart regions to further investigate the role of DNA methylation in functional compartments of the heart.
Sample material encompassed genomic DNA of 68 heart tissue samples from patients with different types of CHD, comprising samples from left and right atrium (LA, RA), left and right ventricle (LV, RV), and the interatrial septum (IAS). Furthermore, we assessed nine controls from non-failing heart samples, as well as induced pluripotent stem cell (iPSC)-derived cardiomyocytes to evaluate the impact of our findings. DNA methylation analysis was done using Illumina's Infinium HumanMethylation450 BeadChip Array. For downstream verification of identified differentially methylated loci, mass spectrometric Epityper technique and bisulfite pyrosequencing has been applied.
Principal component analysis revealed a distinct pattern of atrial (LA, RA, IAS) and ventricular (LV, RV) samples of affected individuals. Overall, 168 significantly differentially methylated loci between atrial and ventricular samples have been detected, showing methylation differences of greater than 30 %. Most of these loci exhibited hypomethylation of atrial samples compared to ventricular samples. These results could be verified by aforementioned techniques and independently validated in the control samples. Interestingly, the iPSC-derived cardiomyocytes showed a methylation pattern resembling the ventricular samples.
In summary, the results of this study suggest that different heart regions, in particular atrium and ventricle, could be distinguished by a specific DNA methylation pattern. To further validate these observations, we are currently testing additional heart tissue samples in an independent cohort, as well as samples from variously differentiated iPSC-derived cardiomyocytes. Overall, our study could provide novel insights for the improvement of *in vivo* differentiation and characterization of cardiomyocytes.

23. Myomesin 2 is involved in assembly of the cardiac sarcomeres during zebrafish embryonic development

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Congenital heart defects (CHD) are the most common congenital malformations in newborns and are a major cause of infant morbidity and mortality. Despite many genetic studies, much of the genetic mechanisms behind normal and abnormal heart development remain to be elucidated.

The myomesin 2 gene (*MYOM2*) encodes a protein located in the M-band of the sarcomere in cardiac and skeletal muscle. A potential role for MYOM2 in the assembly of sarcomeric t itin has been proposed. The assembly of the sarcomere is of tremendous importance for a fully functional skeletal and cardiac muscle and mutations in *ACTC1*, *MYH6* and *MYH7* have previously been associated with CHD and rare *MYOM2* variants have been reported in CHD patients.

A patient with an atrial septal defect was found to have an inversion on chromosome 8 affecting *MYOM2*. This led us to investigate if MYOM2 is expressed in human fetal hearts during development. The zebrafish genome encodes a *myom2* gene with a 55% homology to the human MYOM2. Therefore the zebrafish was used to model the expression of *myom2* during development in whole embryos and to explore the role of gene knockdown in zebrafish.

In situ hybridization showed that *myom2* is expressed specifically in heart and skeletal muscle during zebrafish embryonic development. Morpholino knock down of *myom2* caused defects in heart development, leading to an enlarged heart at 3 dpf. Zebrafish carrying a nonsense mutation in the *myom2* gene also presented with an enlarged ventricle at 3 dpf. Mutant fish, however, displayed normal heart rate and ventricular contraction (as measured by fractional shortening). Electron microscopy analysis of the myocyte ultrastructure, showed disassembly of the cardiac sarcomere at 3 dpf in mutant fish. In skeletal muscle, no difference in ultrastructure was observed between mutant and wild-type fish.
Our study suggests that myomesin 2 plays a crucial role in assembly of the cardiac sarcomeres during heart development.

24. RNA Sequencing of hemodynamically altered hearts reveals differential expression of conduction, structural and metabolic genes

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Structural heart defects and abnormal heart hemodynamics are known to effect conduction [ref 1]. Haemodynamic alteration by outflow tract (OFT) banding has been seen to give rise to precocious development of mature apex to base conduction [ref 2], but little is known about embryonic conduction gene expression in the heart following OFT banding. OFT-banding was performed on chick embryos at HH21, prior to migration of cells of the sinus venosus to the eventual sinoatrial node, and harvested at HH29 when sinoatrial node development is near completion. Global gene expression was assessed by RNA sequencing in banded and control hearts (three per group). OFT banded embryos showed an enlarged heart structural phenotype. The mRNA globin content of blood can measure 75 - 90 % of total transcripts [ref 3]; to enable low gene transcript reads to be assessed this mRNA was removed, giving a >99 % reduction of the selected globin gene mRNA. To our knowledge, globin depletion has not previously been performed in the chick embryo. Sequencing revealed significant (<0.05 corrected $\log_2$ FC) differentiation in 30 genes. 14 of these genes were verified by qPCR and showed a positive correlation ($r(12)=0.98$, $p=<.001$). The verified genes of interest had a metabolic, progenitor, structural or conduction function. We have shown that genes of low read count can be analysed in embryonic chick hearts with prior globin depletion treatment. This enabled hearts with alteration in hemodynamics, and subsequent enlarged structural phenotype, to display differential regulation of key conduction genes as well genes involved in structure, differentiation and metabolism.

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25. The Early Role of Rho kinase (ROCK) in the development of the ventricular wall and cardiomyopathy

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Congenital heart disease is extremely common and accounts for a third of all congenital malformations. In addition, adult heart disease is the main cause of death in the UK. Defects acquired during development may predispose to disease later in life and have a detrimental lasting effect on heart function. Therefore understanding the underlying mechanisms involved in cardiac development and disease progression is extremely important.
The serine threonine kinase, Rho Kinase (ROCK) is the main effector of the small GTPase RhoA and it is involved in a number of diverse cellular functions including regulation of cell morphology, migration, proliferation, apoptosis and polarity as well as being a key regulator in actin-myosin contraction. ROCK is required for normal heart development; however the exact function of ROCK in the cardiomyocytes is unknown.
Conditional downregulation of ROCK specifically in the heart using Cre-LoxP technology has allowed for the investigation of ROCK specifically in the developing cardiomyocytes. ROCK downregulation in the epicardium and myocardium during development results in heart defects, which include an abnormally thin myocardium and persisting trabeculae. Analysis at the cellular level indicates abnormalities in sarcomere assembly in mutant hearts which are present from early in development. Interestingly these mice survive into adulthood where they develop characteristics associated with cardiomyopathy including hypertrophy, fibrosis and a reduction in heart function. This model highlights the importance of understanding developmental defects and how they contribute to adult disease. This model will help in identifying cellular mechanisms underpinning the development of adult cardiovascular disease.

26. **MicroRNA biogenesis is dysregulated in Idiopathic Dilated and Chagas Cardiomyopathies**

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Chagas disease results from *Trypanosoma cruzi* infection, which may lead to the chronic Chagas cardiomyopathy (CCC), a dilated cardiomyopathy characterized by myocarditis, hypertrophy and fibrosis. Studies suggest that CCC has a worse prognosis than other cardiomyopathies such as Idiopathic Dilated Cardiomyopathy (DCM). MicroRNAs, posttranscriptional regulators of gene expression, have recently been shown by our group to be downregulated in the heart of patients with DCM and CCC, being involved in the regulation of several processes such as cell proliferation, fibrosis and repair. Since all muscle-specific miRNAs (myomiRs) were downregulated, we hypothesized that a dysregulation in the microRNA processing (biogenesis) machinery could be responsible for this finding. To evaluate our hypothesis, RT-qPCR and western blot were performed in 37 heart samples (6 controls, 16 CCC, 15 DCM) to analyze heart-specific miRNA primary transcripts expression (primiR-208a/b, -133a/b, -1, -126, -367, -302a-d) and mRNA and protein expression of the main proteins involved in miRNA biogenesis: Dicer, Drosha, DGCR8, Exportin 5, TRBP, PACT and AGO2. Our preliminary data reveal a downregulation of TRBP (FC: -9.3 for DCM, $p<0.01$), Exportin 5 (FC: -1.3 for CCC, $p<0.05$) and DGCR8 (FC: -1.4 and -1.9 for CCC and DCM, respectively; $p<0.05$) mRNAs with different expression patterns between the two cardiomyopathies. Although Dicer mRNA was not significantly dysregulated, Dicer protein expression was found to be downregulated in both clinical groups ($p<0.01$), which suggests a possible posttranslational interference leading to a global miRNA dysregulation. No significant differences in the expression of heart-specific primiRNAs were found for any of the clinical groups when compared with controls. These results support the idea that the downregulation of mature myomiRs is not related to an altered transcription but to a biogenesis dysregulation, which may be a crucial step in the development of heart pathologies.

27. Expression of the Wilms tumor suppressor gene (*Wt1*) in a subpopulation of embryonic cardiomyocytes is required for cardiac development

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The Wilms' tumour gene, WT1, encodes a zinc-finger transcription factor involved in the development of several organs. WT1 is expressed during mammalian embryonic development in many tissues, including the urogenital system, spleen, spinal cord, diaphragm, coelomic epithelium and epicardium (Armstrong et al., 1993; Moore et al., 1999; Cano et al., 2016; Ariza et al., 2016; Carmona et al., 2016). Post-transcriptional modifications of the Wt1 pre-mRNA lead to the production of up to 24 different isoforms, which seem to serve distinct but overlapping cellular and developmental functions.

We have checked if Wt1 is expressed by the embryonic myocardium. Using transgenic mice lines for lineage tracing (mWt1/IRES/GFP^{Cre};ROSA26R^{EYFP}), we have detected a small population of cardiomyocytes from a Wt1-expressing cell lineage in early developmental stages (E8.5-E9.5). These cardiomyocytes were mainly located in the inflow tract, but some of them were observed in the ventricles. We confirmed Wt1 expression by RT-PCR in hearts before the attachment of proepicardial cells, when the cardiac tube is only constituted of myocardium and endocardium.

We have also studied the mRNA levels of the four main isoforms of Wt1 in a reverse transcriptase-polymerase chain reaction (RT- PCR) assay. We have found differential expression of these Wt1 isoforms in the embryonic heart. Conditional deletion of Wt1 in cardiac Troponin T expressing cells caused severe damage in the developing heart, particularly muscular defects in the interventricular septum and free ventricular walls, as well as defective sinus venous formation. These embryos did not survive after birth. Likewise, conditional deletion of GATA4 in Wt1-expressing cells causes a similar phenotype in the myocardium, but also defects in the proepicardium, epicardium and subepicardial space, causing embryonic death around E11.5. Thus, we conclude that Wt1 is expressed in a subpopulation of early embryonic cardiomyocytes, and this expression seems to be essential for heart development.

28. Evaluation of the zebrafish desmin gene duplication event

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Background: Mutations in the type III intermediate filament, desmin, are associated with limb-girdle muscular dystrophy and cardiomyopathy. The mechanism of the late onset myopathy and variable cardiac phenotype remain unclear. Although there are a limited number of transgenic and knockout mice models, there is a need for a tractable *in-vivo* laboratory model to investigate these aspects. The ease of genetic manipulation and the vertebrate body plan of the zebrafish suggests that this would be a candidate system. However, zebrafish have undergone a genome duplication event, which complicates their use fro genetic studies.

Aim: We set out to evaluate the effect of genome duplication on the expression of the desmin gene in zebrafish.

Method: The desmA and desmB transcripts were cloned and compared to human desmin. We then mapped the expression pattern of the desmin A (desmA) and desmin B (desmB) genes through development and in adult fish using RT-PCR and *in-situ* hybridization. Desmin A and Desmin B mutant lines have been generated by CRISPR-Cas9 genome editing.

Results and Conclusion: We demonstrate differential expression and co-expression of the two desmin gene products during development and in adult zebrafish.

29. SMURF1 is expressed in SHF structures and may serve as negative regulator of cardiomyogenesis in SHF cells

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We have investigated the role of the E3 ubiquitin ligase SMURF1 in human and mouse heart development, and in *in vitro* differentiation of P19.CL6 stem cells and mouse embryonic stem cells (mESC) into cardiomyocytes.

Immunohistochemical and qRT-PCR analyses on human embryonic and fetal heart tissues showed that SMURF1 has a distinctive spatiotemporal expression pattern, suggesting that SMURF1 is important for development of secondary heart field (SHF) structures, including the outflow tract (OFT) and the right ventricle. Furthermore, SMURF1 is expressed in the conduction system. Smurf1 is also expressed in the murine heart, and Smurf1 knockout mice display a delay in septation of the outflow tract at E12.5, supporting a role of Smurf1 in formation of SHF structures.

To characterize the role of SMURF1 during cardiomyogenesis, we generated stable P19.CL6 cell lines with CRISPR/Cas9-mediated deletion of *Smurf1* and analyzed the ability of the cells to leave their pluripotent state and form clusters of beating cardiomyocytes. We found that knockout of Smurf1 increases the rate of cardiomyogenesis, suggesting that Smurf1 functions as a negative regulator of cardiomyocyte differentiation. In cultures of cardiomyocytes derived from mESCs, siRNA-mediated knockdown of Smurf1 results in a decrease of cardiac fibroblasts in both SHF and FHF cells, suggesting that the delay in OFT septation in the mouse is due to a decrease in cardiac fibroblasts, although this needs to be investigated in more detail.

In conclusion, we suggest that SMURF1 plays a critical role in heart development - in particular in development of SHF structures by serving as a negative regulator of cardiomyogenesis and a positive regulator of cardiac fibroblasts.

30. Identifying Proteomic Signatures of Heart Development: proteins of the branched-chain amino acid metabolism pathway

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Fetal and adult heart muscles differ markedly at molecular, structural and physiological levels. There is great interest in identifying those differences as in conditions leading to heart failure the adult heart reverts towards a fetal-like gene expression programme. Here, we have performed a proteome-scale comparison of fetal and adult guinea pig (1) ventricular tissue to identify proteins and pathways involved in heart development/maturation.

Adult female (pregnant and non-pregnant) guinea pigs were killed according to Home Office guidelines. Fetal (gestation day 65-67, term ~day 67) and adult left ventricle tissues were homogenised in two separate extraction buffers to accommodate the large dynamic range of protein expression. Protein lysates were treated with trypsin and analyzed using two separate LC-MS instrument platforms. Raw data was searched against an in-house customised guinea pig database. >3000 proteins (>25,000 peptides) were identified and >2,000 quantified.

The expression of 189 proteins was raised in adult, and 489 in fetal, heart muscle. Pathway analysis revealed that a prominent feature of adult tissue was the increased expression of many proteins essential for the intracellular degradation of the branched-chain amino acids (BCAA) leucine, isoleucine and valine. BCAAs play key roles in protein synthesis, cellular growth and metabolism and up-regulation of proteins involved in their catabolism likely reflects the maturation of cardiomyocyte phenotype. BCAA catabolism pathway proteins increased included: branched chain amino acid transaminase (BCAT2), branched chain keto-acid dehydrogenase (BCKDHA/B), propionyl-CoA carboxylase (PCCA/B) and methylmalonyl-CoA mutase (MUT). Of note genetic disorders of PCCA/B and MUT are associated with cardiomyopathies (2) and suppression of BCAA degradation has been associated with a murine model of heart failure (3). These data suggest that regulating the expression of proteins key to BCAA degradation is important for cardiac maturation and identifies these molecules as interesting candidates to study in the progression (and possible treatment) of cardiopathologies.

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2. Huang et al., (2011) Branched chain amino acid metabolism in heart disease: Cardiovasc. Res. 90: 220-223
3. Sun et al., (2016) Catabolic Defect of Branched-Chain Amino Acids Promotes Heart Failure. Circulation 133: 2038-2049

31. An investigation into the role of Rac1 during ventricular development

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Background: Rac1 is a member of the Rho family of small GTPases. In *Rac1* knock-out mice, the heart morphology is altered, with a dilated, thin myocardium and septal defects. The role of *Rac1* in ventricular development during cardiac morphogenesis is poorly understood. We hypothesis that Rac1 mediates the regulation of actin organisation and therefore morphogenesis of the heart in particular during compaction of ventricular myocardium.

Aims: The overall aim is to determine the role of *Rac1* in cardiomyocytes using a cardiomyocyte specific transgenic mouse model, *TnT-Cre*. We will investigate protein expression during ventricular development of *Rac1;TnT-Cre* embryos, evaluating the role of the extracellular matrix, the endocardium, the epicardium and Rac1 interactors associated with ventricular development.

Method: Using *Cre-lox* technology we created a cardiac-specific *Rac1* knock-out in cardiomyocytes from embryonic day (E) E7.5 by crossing $Rac1^{F+}$*;TnT-Cre* males with *Rac1* floxed females ($Rac1^{FF}$). Using immunohistochemistry protein expression pattern was characterised and compared in, three somite, and litter matched control and mutant embryos at E10.5 and E11.5.

Results: Downstream disruption of the actin organisation impacts on the arrangement of cardiomyocytes. Consequently, this results in disorganisation of the endocardium, leading to pooling of endothelial cells with associated extracellular matrix changes. During normal development, Mena, a Rac1 interactor, becomes restricted to the trabeculae, however in *Rac1;TnT-Cre* embryos Mena appears dysregulated, with continued expression in compact myocardium. The absence of Rac1 in the myocardium triggers compensatory pathways such as the redistribution of Vegfr2 and Vav2.

Conclusion: In *Rac1;TnT-Cre* embryos the transition from E10.5 to E11.5 is a key stage where by lack of *Rac1* leads to abnormal localisation and dysregulation of actin organisation, and consequent defects in spatiotemporal arrangement of cells within the myocardium. In normal development, active Rac1 is essential for the appropriate localisation of Mena and actin organisation, which is essential for the normal maturation of the cardiomyocytes. The altered actin cytoskeleton and extracellular matrix leads to abnormal morphology of the cardiomyocyte. The change in morphology of cardiomyocytes leads to a loss of their directionality and fail to form normal trabeculae and compact layer.

32. Reversing *mdx* cardiomyocyte hypertrophy *in vitro*

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Several promising therapies aimed at treating Duchenne Muscular Dystrophy (DMD) are under investigation at various pre-clinical and clinical stages. Unfortunately many of these approaches, including exon skipping and viral-mediated gene therapy, have shown to be more efficient in skeletal muscle than in the heart. In addition to this, there is a growing need to treat dystrophin-deficient cardiomyopathy as patient life expectancy is increasing and symptoms of cardiomyopathy in the patient population are becoming more prevalent. Despite the primary genetic defect being identical in skeletal and cardiac muscle, the symptoms and severity often differ suggesting the involvement of secondary organ-specific pathways that are yet to be fully understood. In order to more efficiently screen treatments in the dystrophic heart, we have developed an *in vitro* model of cardiomyocyte hypertrophy using primary embryonic cardiomyocytes isolated from *mdx* hearts. Using this newly developed model, we have been able to utilise various pharmacological and gene therapy approaches to decrease the hypertrophic phenotype seen in the model. This model could be a fast and efficient way to screen new and existing pharmaceutical compounds in addition to gene editing and gene replacement approaches for potential therapeutic efficacy in dystrophin-deficient cardiomyopathy. In addition to the screening of therapeutic compounds, using transcriptomics and proteomic approaches, this model can be used to deepen our understanding of the secondary organ-specific pathways involved in the pathophysiology of the heart in DMD and potentially identify novel therapeutic targets. RNA-sequencing of hypertrophic *mdx* cardiomyocytes identified differential regulation of genes and pathways both across the time course of the hypertrophic response and in comparison to wild-type controls. Identified genes include those involved in calcium ion handling, fibrosis and angiogenesis.

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