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Essential role of GATA5 in the mammalian heart

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Essential role of GATA5 in the mammalian heart

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Résumé

Chez l'humain, les maladies congénitales cardiaques (MCC) sont présentes chez 3-4% des nouveaux nés et sont une cause importante de mortalité infantile et de morbidité dans le monde. La majorité des MCCs implique les valves et les septums, qui proviennent des cellules endocardiques. Les valves aortiques bicuspides (VAB) sont la MCC la plus fréquente chez l'humain, avec un taux estimé de 1-2% dans la population. Cependant, les gènes et les mécanismes moléculaires qui causent cette malformation demeurent obscures. Le facteur de transcription GATA5 est exprimé dans les cellules et les coussins endocardiques de façon transitoire durant la septation et la formation des compartiments cardiaques. Chez le poisson zèbre, des mutations dans le gène *Gata5* causent des malformations cardiaques sévères incluant l'absence de cellules endocardiques. *In vitro*, l'inhibition de *Gata5* bloque la différentiation endocardique. Ces études suggéraient donc un rôle important de GATA5 dans la formation du cœur.

Dans le cadre de ce projet de doctorat, nous avons analysé le rôle de GATA5 dans le développement du cœur en produisant des lignées de souris chez lesquelles le gène *Gata5* était inactif soit dans toutes les cellules ou uniquement dans les cellules endocardiques. Les souris possédant 2 allèles mutées du gène *Gata5* étaient viables mais plus de 26% des souris $Gata5^{-/-}$ ont développé des VABs. Par ailleurs, une incidence similaire de VABs a été obtenue chez les souris ayant une délétion spécifique de *Gata5* des cellules endocardiques, obtenue en croisant les souris $Gata5^{WT/Flox}$ avec les souris transgéniques *Tie2-Cre*. Sur le plan mécanistique, une réduction significative de JAG1, un corécepteur pour Notch1, ainsi qu'une augmentation marquée de Rbj un répresseur de cette voie, ont été détectés chez les souris $Gata5^{-/-}$ et *Tie2*-cre+;$Gata5^{Flox/Flox}$, suggérant qu'une dérégulation de la voie Notch dans les cellules endocardiques puisse être la cause des VABs. Ces résultats démontrent l'importance de GATA5 pour le développement endocardique et la formation de la valve aortique. De plus, ils identifient GATA5 comme gène candidat de MCCs chez l'humain.

Environ 12-14% des MCCs sont causés par le développement anormal de la voie de chasse, menant aux malformations telles que la transposition des grandes artères, la tétralogie de Fallot ou le syndrome du ventricule droit à double issue. Des mutations dans *Gata4* et *Gata6* sont associés à des défauts de la voie de chasse, dans

plusieurs espèces incluant l'humain. Nous avons examiné si GATA5 interagit avec GATA4 ou GATA6 dans le développement de la voie de chasse. Alors que les souris hétérozygotes pour *Gata5*, *Gata4* ou *Gata6* ont des défauts cardiaques subtiles et sont viables, les embryons *Gata4*$^{+/-}$*Gata5*$^{+/-}$ et *Gata5*$^{+/-}$*Gata6*$^{+/-}$ démontrent une létalité embryonnaire et périnatale due à des défauts cardiaques, tel qu'un ventricule droit à double issue et des défauts de septation ventriculaire. Ces résultats indiquent l'importance des interactions génétiques entre GATA5 et les autres facteurs GATA pour la rotation et l'alignement de la voie de chasse au cours du développement cardiaque et soulèvent la possibilité que des changements subtiles de l'activité de 2 facteurs GATA puissent mener à des MCCs chez l'humain.

Mots-clés : maladie congénitale cardiaque, valve aortique bicuspide, voir de chasse, GATA, développement cardiaque, septation, ventricule droit à double issue, coussin endocardique, facteur de transcription.

Abstract

Congenital heart defect (CHD) in humans occur in 3-4% of live birth and is a major cause of infant mortality and morbidity in the world. The majority of CHD involves the valves and septa, which originate from endocardial cells. Bicuspid aortic valve (BAV) is the most common CHD in humans with an estimated rate of 1-2% in the population. However, very few genes have been linked to this defect and the mechanisms underlying BAV formation remain undefined. GATA5, a member of the GATA family of transcription factors, is expressed in a spatial and temporal manner in the developing heart where it is predominantly found in endocardial cells and endocardial cushions (ECs) of the outflow tract (OFT) and atrioventricular canal between E9.5-E12.5 in the mouse. Mutations in the *Gata5* gene in zebrafish (*faust* mutants) cause cardia bifida and lead to endocardial cell depletion. *In vitro* studies using antisense mRNA against *Gata5* revealed a critical role for this gene in differentiation of endocardial cells.

In the context of the present doctoral research project, we investigated the role of GATA5 in mammalian heart development by generating a mouse line with a null *Gata5* allele. *Gata5* null mice are viable but over 26% of them developed BAVs. Endocardial specific deletion of *Gata5* obtained by crossing mice with floxed (Flox) *Gata5* alleles with *Tie2*-cre transgenic mice resulted in a similar incidence of BAVs. RNA profiling revealed that *Jag-1*, a co-receptor for *Notch1*, is significantly downregulated in both *Gata5* null and *Tie2*-cre+;$Gata5^{Flox/Flox}$ mice, suggesting that disruption of Notch signaling in endocardial cells may be the underlying mechanism of disease. These findings reveal an important function for GATA5 in endocardial cell development and aortic valve formation and identify GATA5 as an important candidate CHD causing gene.

Abnormal development of the OFT accounts for about 12-14% of all CHDs, leading to malformations such as persistent truncus arteriosus (PTA), tetralogy of Fallot (TOF), double outlet right ventricle (DORV) and transposition of the great arteries (TGA). Both GATA4 and GATA6 play important role in OFT development. We tested whether GATA5 might interact genetically with GATA4 and GATA6 for proper heart morphogenesis. We found that, whereas mice lacking a single copy of *Gata5*, *Gata4* or *Gata6* have subtle cardiac defects, the $Gata4^{+/-}Gata5^{+/-}$ and $Gata5^{+/-}Gata6^{+/-}$ mutant

embryos show embryonic and perinatal lethality due to severe heart defects, including double outlet right ventricle and ventricular septal defects. These findings reveal the importance of genetic interactions between GATA5 and the other cardiac GATA factors in the normal rotation and patterning of the OFT during heart development *in vivo*. The results raise the possibility that subtle alterations in the level or activity of 2 cardiac GATA factors might lead to congenital heart disease in human.

Keywords : Congenital heart disease, bicuspid aortic valve, GATA, cardiac development, double outlet right ventricle, outflow tract, septation, endocardial cushion, transcription factor

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Abbreviations

ANF	Atrial natriuretic factor
AS	Aortic stenosis
ASD	Atrial septal defect
AV	Atrioventricular
AVC	Atrioventricular canal
AVSD	Atrioventricular septal defect
BAV	Bicuspid aortic valve
BMP	Bone morphogenetic protein
BNP	Brain natriuretic factor
CAVC	Complete atrioventricular canal
CHD	Congenital Heart disease
CCS	Cardiac conduction system
CNC	Cardiac neural crest
DORV	Double outlet right ventricle
DPP	Decapentalplegic
EC	Endocardial cushion
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transformation
EPDC	Epicardium derived cells
ES	Embryonic stem
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FOG	Friend of GATA
HCM	Hypertrophic cardiomyopathy
Hh	Hedgehog
HOS	Holt Oram syndrome
HSC	Hematopoietic stem cell
IVS	Interventricular septum

LPM	Lateral plate mesoderm
L-R	Left-right
LVPW	Left ventricular posterior wall
MEF2	Myocyte enhancer factor 2
NC	Neural crest
NFATc	Nuclear factor of activated T cells
NOS	Nitric oxide synthase
Nppa	Natriuretic peptide precursor A
Nppb	Natriuretic peptide precursor B
NRG	Neuregulin
OFT	Outflow tract
PAS	Primary atrial septum
PFO	Patent foramen ovale
PR	Parietal ridge
PS	Pulmonary stenosis
PTA	Persistent truncus arteriosus
RA	Retinoic acid
SA	Sinoatrial
SHF	Secondary heart field
Shh	Sonic hedgehog
Smo	Smoothened
SR	Septal ridge
SRF	Serum response factor
TGA	Transposition of the great arteries
TOF	Tetralogy of Fallot
TGFβ	Transforming growth factor beta
VEGF	Vascular endothelial growth factor
VSD	Ventricular septal defect
VSM	Vascular smooth muscle

To Arianne, my god-daughter, who died of congenital heart disease at the age of 7, you were my light, my sunshine, my joy of life, you will always be in my heart

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Chapter 1

Introduction

1.1. Heart development

In humans, congenital heart disease (CHD) is estimated to occur at an incidence of 4-5% of live births and represent 25% of all congenital malformations. Eventhough important advances have been made in the diagnosis, surgical repair and therapeutic interventions, CHD still leads to significant mortality and morbidity in the human population. CHD is a heritable trait but, the genetic basis underlying the majority of cardiac malformations remains poorly understood. In the past decade, thanks to the use of animal models as well as genetic and biochemical approaches, a large numbers of genes have been discovered to play important roles in diverse aspects of cardiac development. This, in turn, has greatly increased our understanding of the different steps of cardiac morphogenesis and identify CHD causing genes in human.

1.1.1. Cardiac induction

The heart is the first organ that forms in vertebrates. Its development begins with the specification of the cardiogenic mesoderm, followed by determination and patterning of the bilateral heart fields as well as cardiomyocyte differentiation and formation of the heart tube (Figure 1.1). This section focuses on the specification of the cardiogenic mesoderm as well as the inductive signals that regulate this process.

1.1.1.1. Formation of the pre-cardiac mesoderm

The mesodermal tissue that gives rise to the heart first becomes evident when the embryo is undergoing the process known as gastrulation, which occurs between embryonic day (E) 6.5 and E7.5 in the mouse and between stages Hamburger-Hamilton (HH)3 and HH5 in the chick (1;2). At the time of implantation in the mouse (E3.5-E4.5), the inner cell mass, composed of the epiblast and primitive endoderm, and the polar trophectoderm grow into the blastocyst cavity to form a cylinder that adopts an asymmetrical curvature on its anterior and posterior side (future anterior-posterior axis). Soon after the primitive endoderm (in the mouse) layer or the hypoblast (in the chick) is completed, posterior epiblast cells move into the midline where they undergo an epithelial-mesenchymal transition, resulting in the formation of the primitive streak (PS) in the mouse and chick. During gastrulation, the main germ layers will be established: (1) the ectoderm, which gives rise to the skin and nervous system, (2) the mesoderm,

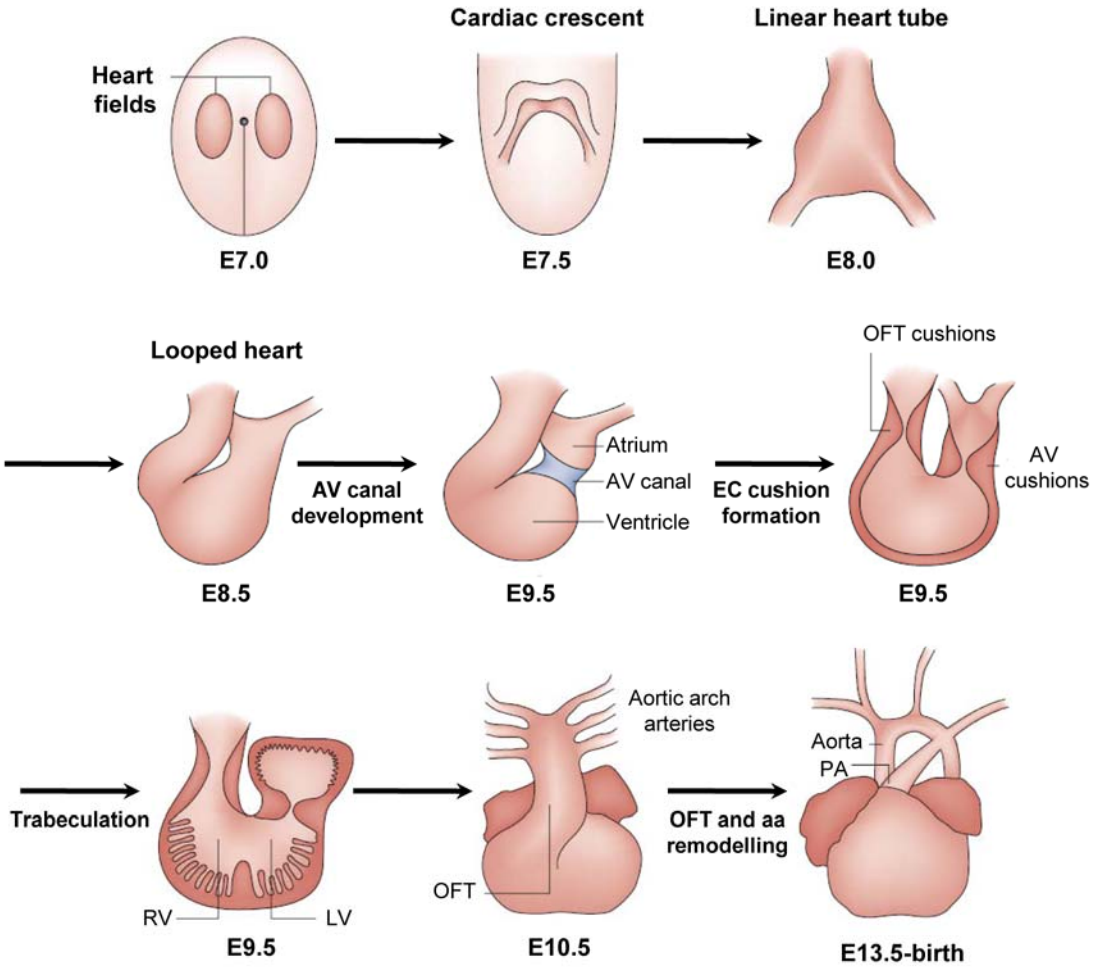


Figure 1. 1. Stages of cardiac development

Heart development in the mouse. AV: atrioventricular; LV: left ventricle; PA: pulmonary artery; OFT: outflow tract; RV: right ventricle. Adapted from High F and Epstein J, *Nature Review Genetics*, 2008; 9 (49-61).

which is required for the formation of muscles, and (3) the endoderm, which gives rise to the gut and associated organs. It is during gastrulation that cardiac progenitor cells will ingress into the cranial portion of the PS and start to migrate anterolaterally to form separated but paired left and right heart-forming region in the anterior lateral plate mesoderm (2;3). Epiblast explants studies or heterotypic transplantation of the PS have revealed that cells are specified but not determined to a myocardial fate until after their ingression into the PS and during their migration (4).

Previous fate-mapping studies have identified the epiblast as the major source of embryonic cardiac mesoderm, which is located just lateral to the primitive streak and caudal to the node (5;6). In the mouse, the earliest heart precursors can be traced to about 50 founder cells located on both sides of the midline in the epiblast (1). This localization of cardiac progenitors is supported by explantation studies where the posterior epiblast has cardiogenic potential and will differentiate into cardiomyocytes in a defined culture medium (7). Similar results were obtained after transplantation of epiblast-derived cells to the cardiogenic field of the late primitive streak stage, suggesting that ingression is not required for myocardial fate (1;8). Location of the heart precursors in the cardiac mesoderm at E7.0-7.5 coincides with expression of *Gata4*, *Flt-1* and *Nkx2.5* genes (9;10).

1.1.1.2. Signalling pathways involved in induction of precardiac mesoderm

Signals emanating from the endoderm have been shown to be essential for cardiac differentiation in vertebrate species. For example, in the mouse, isolated mesodermal cells at E7.25 fail to differentiate into beating cardiomyocytes but when these explants were isolated with primitive streak and visceral endoderm, differentiation of cardiomyocytes occurred, thus suggesting that the visceral endoderm is required for proper differentiation of cardiac cells (11). Candidates that promote specification of the precardiac mesoderm include members of the transforming growth factor beta (TGFβ) superfamily and fibroblast growth factors (FGFs) whereas members of the canonical Wnt/β-catenin pathway inhibit myocardial induction (2;12).

Evidence for a direct involvement of BMP signalling in induction of the cardiogenic mesoderm was initially obtained through studies of Decapentalplegic (Dpp) in *Drosophila*. Studies of *Dpp* mutant embryos revealed that *Dpp* is involved in the formation of dorsal vessels and induction of *tinman* expression in the dorsal mesoderm

(13). *Dpp* is a member of the TGFβ superfamily and is closely related to BMP2 and BMP4 in vertebrates. Bmp2 and Bmp4, which are secreted by the endodermal cells underlying the mesoderm, seem to be the only BMP isoforms able of inducing the formation of cardiogenic cells in non-precardiac mesoderm (14). Consistent with this, application of BMP2-soaked beads to regions of non-cardiogenic mesoderm *in vivo* results in ectopic expression of key cardiac transcription factors like *Nkx2.5*, *GATA4* and *Tbx2* and *Tbx3* (15). However, addition of noggin, which antagonizes BMP signalling, completed inhibited differentiation of the precardiac mesoderm (14;16). In addition, forced expression of dominant-negative BMP receptor in the developing Xenopus embryo resulted in reduction or absence of heart formation (17). Together, these experiments led to the conclusion that BMP signalling plays an important role in the early steps of cardiogenic induction, inducing mesodermal cells into the cardiogenic lineage and maintaining their cardiogenic potential until later signals complete their differentiation into cardiomyocytes.

Several studies have shown that members of the fibroblast growth factor (FGF) family, including Fgf2, Fgf4 and Fgf8 cooperate with Bmp2 in early cardiogenesis. For example, expression of Fgf8 in the endoderm of mouse embryos is required to induce expression of *Nkx2.5* and *Mef2c* in the precardiac mesoderm and ectopic Fgf8 expression leads to ectopic expression of these cardiac markers (18;19). These studies revealed that maximal induction of cardiogenic cells requires continuous expression of BMP whereas FGF is only required transiently. Overall, it has been suggested that full cardiogenic potential in the heart forming fields requires BMPs to induce mesodermal cells and FGFs for the subsequent proliferation and survival of the differentiated cardiomyocytes.

Studies of cardiac development in *Xenopus* and chicken revealed that some canonical Wnt ligands that activate the Wnt/β-catenin pathway can repress cardiogenesis. Specifically, injection of Wnt3A and Wnt8 into the cardiogenic dorsal zone explants in *Xenopus* blocks expression of Nkx2.5 and Tbx5, thereby inhibiting cardiac induction (16). Similarly, ectopic expression of Wnt3A or Wnt1 in chick embryos blocked cardiac gene expression in the precardiac mesoderm (20). Conversely, when the canonical Wnt pathway was inhibited by injection of GSK3β, induction of cardiac mesodermal progenitors occurred (16). Thus, induction of cardiac morphogenesis requires inhibition of the Wnt pathway and antagonist Wnt candidate

molecules have been identified in both chick and frog. Indeed, it has been shown that Dickkopf (Dkk-1), Crescent and XDbf4 play important roles in establishing the precardiac mesoderm through inhibition of specific Wnt ligands (16;20;21). More recent studies suggest substantial overlap between the noncanonical and canonical Wnt/β-catenin pathway (22;23). A role for Wnt11 in cardiac development was inferred based on its expression in the embryonic mouse myocardium and in differentiating embryoid bodies, at the same time as *Nkx2.5* (24;25). Both gain and loss of function studies in chick and Xenopus confirmed that Wnt11 is a potent inducer of the early heart field (26;27). Similarly, ectopic administration of Wnt11 in mouse ES cells or P19 cells is able to induce expression of early cardiac markers such as *Nkx2.5* and *Gata4* (25;27). Lastly, it has been proposed that activation of protein kinase C or CamkII by the Wnt/Ca2+ pathway inhibits the canonical Wnt signalling either upstream or downstream of β-catenin, providing a means to regulate cardiac induction (28).

1.1.2. Linear heart tube formation

As described in the previous section, cardiac progenitors arise in the mesodermal layer, which forms by ingression of cells into the primitive streak. At around E7.0-7.5 in the mouse and stage 3 in the chick, the cardiac progenitors will leave the PS and migrate both laterally and anteriorly to reach the head fold on either side of the ventral midline, forming the cardiac crescent. At that stage, two different heart fields can be distinguished. Labelling analysis of heart progenitor cells in chick have revealed that these cells intermingle during their migration from the PS; this is in contrast to early studies, which suggested that these cells behave as a coherent sheet (29). Similarly, in the mouse embryo, retrospective clonal analysis of myocardial cells demonstrated that their precursors follow a proliferative mode of growth, with an initial dispersive phase, followed by coherent cell growth (30). Starting at E7.5 in the mouse embryo, a second wave of migration will bring the cardiac progenitors to the ventral midline, where they will fuse to form a beating linear heart tube by E8.0 (Figure 1.1). This linear heart tube is composed of an outer myocardial layer and an inner endocardial layer, both separated by the extracellular matrix.

Proper migration of the mesodermal cells from the PS to their final position at the ventral midline as well as correct embryonic endoderm differentiation is critical for heart fusion, and mutations of many genes affecting these processes lead to various

degrees of *cardia bifida*, a failure of heart fusion, which results in the formation of two bilateral heart primordia. In zebrafish, the *Casanova/Sox32*, *bonnie-and-clyde* mutants, which fail to form endoderm, display *cardia bifida* (31;32). Similarly, *faust/Gata5* mutants in zebrafish also display *cardia bifida*, but in this case, it results from defective differentiation of the endoderm, which inhibits ventral migration and prevents concomitant movement of myocardial cells (33). In *Casanova* mutants, the heart primordia show correct patterning of atrial and ventricular myocytes whereas this is not the case in *faust* mutants. In mouse, defective ventral morphogenesis and foregut formation, which is observed in *Gata4* and *Foxp4* null embryos, has also been linked to the formation of two heart primordial (34-36). In the *Foxp4* mutants, in contrast to *Gata4* null embryos, each heart-forming field was able to develop into a differentiated four chambered heart. The bHLH transcription factor *Msp1* is an early cardiac progenitor marker and is required for their migration to the midline. In the absence of *Mesp1* in mouse embryos, cardiac progenitors migration is delayed, resulting in partial or complete *cardia bifida* (37).

1.1.2.1. The heart fields

Pharyngeal mesoderm derived cells contribute to the myocardium at the arterial pole of the heart tube (38-40). The origin of these cells was termed the anterior heart-forming or secondary heart field (SHF). Waldo *et al* used cell labelling and quail-chick chimeras to determine the origin of the outflow tract (OFT) myocardial progenitors. After observing that the heart field markers *Nkx2.5* and *Gata4* were located in the pharyngeal mesoderm at HH14 in chick, they labelled this region, which was subsequently found in the developing OFT at HH22. Mjaatvedt *et al*, also using chick as model system, labelled myocardial progenitors to locate the origin of cells incorporated in the OFT (39). These fate mapping experiments showed that cells labelled in the mesoderm surrounding the aortic sac and anterior to the primitive right ventricle are incorporated into the OFT. Moreover, when the bilateral heart fields were ablated, the embryos only formed a rudimentary heart tube, which suggested that the OFT progenitors derived from a separate population of cells than the bilateral fields. Finally, studies in mice also lead to the identification of the secondary heart field (38). Kelly *et al* created an enhancer trap transgene that initially showed expression in part of the pharyngeal mesoderm. Cells that had expressed the transgene were subsequently shown to contribute to the myocardium of the arterial pole of the heart. Using DiI

labelling, the authors showed a progressive ventral movement of pharyngeal mesoderm cells into the lengthening heart tube. A contribution of this anterior heart field to the right ventricle as well as the OFT myocardium was demonstrated using DiI labelling and explants experiments (41). Waldo *et al* extended these observations by showing that cells of the SHF also contribute to the formation of smooth muscle cells that form the proximal walls of the aortic and pulmonary trunk (42).

Thus, once formed, the linear heart tube elongates by addition of cells at the venous and arterial poles. The cells that make up the primitive heart tube are derived from the primary heart field, which contributes to the left ventricle and ventricular septum of the adult heart (Figure 1.2) (41;43). The cells that are added at the venous pole will give rise to the myocardium of the AV canal, atria and inflow tract. Cells of the SHF, on the other hand, will be added at the arterial pole of the heart and will contribute to the ventricular septum, right ventricle and outflow tract (Figure 1.2). The discovery of the SHF has considerably advanced our understanding of cardiac development, especially concerning early heart morphogenesis. Cells located in the pharyngeal mesoderm are distinguished by the expression of *Islet1* (*Isl1*), *Fgf8* and *Fgf10* (38;44;45).

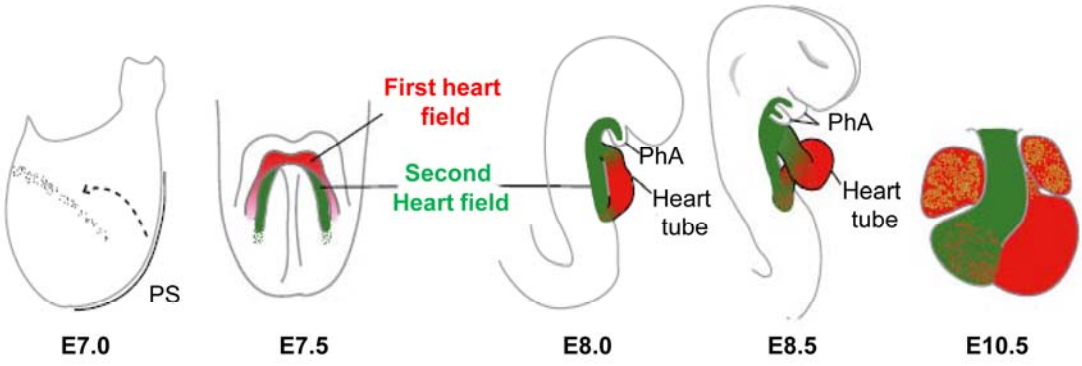


Figure 1. 2. Contribution of the heart fields to the developing heart

Relative position and contribution of the SHF progenitors (green) relative to the FHF (red) from the cardiac crescent at E7.5 to the looping stages of the mouse heart at E8.5. PhA: pharyngeal arches; PS: primitive streak. Adapted from Meilhac S and Buckingham M, the behaviour of cells that form the myocardial compartment of the vertebrate heart, in *Cardiac development and regeneration*, 2010: 195-217.

1.1.2.2. Molecular patterning of the secondary heart field

As the SHF cells contribute to the heart, they are exposed to signals and patterned by signalling pathways and transcriptional regulators. This signalling network plays important roles in anterior/posterior and left/right patterning of the SHF, in maintenance of proliferation, differentiation and in interaction with other cardiac cell lineages.

1.1.2.2.1. Retinoic acid signalling in A/P patterning

Retinoic acid (RA) signalling in the posterior region of the heart is required for SHF development. The effects of excess or reduction of RA on cardiac morphogenesis has been demonstrated in diverse animal models (46). Generation of a hypomorphic allele for the RA-synthesizing enzyme retinaldehyde dehydrogenase 2 (*Raldh2*) leads to perinatal death and exhibit features of the DiGeorge syndrome, in which *Tbx1* is implicated (47). Analysis of *Tbx1/Raldh2* compound mutants demonstrated that decreases in the levels of RA accelerates the recovery from arterial growth delay seen in *Tbx1*-null embryos (47;48). *Raldh2* null mouse embryos display severe RA deficiency and cardiac defects characterized by absence of heart looping, impaired sinoatrial growth and defective ventricular trabeculation (49;50). Furthermore, loss of *Raldh2* is also associated with abnormal posterior expansion of SHF markers including *Tbx1*, *Fgf8* and *Fgf10*, suggesting that RA signalling limits the extent of SHF (51;52). These observations indicate that RA signalling is required to delimit the domain of mesoderm that is competent to become the SHF. Similarly, vitamin A deficiency in avian embryos or addition of RA soaked beads locally downregulates *Tbx1* expression (53). This suggest the existence of a feedback loop between the RA signalling and Tbx1. This is further confirmed in *Tbx1* mutant embryos, where the *Raldh2* expression domain is shifted anteriorly and RA catabolising enzymes like Cyp26 are downregulated (54;55).

1.1.2.2.2. FGF signaling in maintenance of SHF cell proliferation

Several signalling pathways have been shown to regulate proliferation of cells in the SFH, including the FGF, BMP, hedgehog and Wnt pathways. Fgf8 and FGF10 have been extensively studied for their impact on SHF development. *Fgf10* null mice exhibit perinatal lethality due to the complete absence of lungs (56;57). Further analysis of *Fgf10*$^{-/-}$ mice revealed a role for this secreted molecule to correctly position

the heart in the thoracic cavity, however OFT elongation and right ventricle formation appeared normal, suggesting that *Fgf10* mutants do not show an SHF phenotype (58). In contrast to Fgf10, Fgf8 has now been recognized as a major player in SHF development. Mice carrying a hypomorphic allele of *Fgf8* survive to term with abnormal cardiovascular patterning, including OFT misalignement defects, VSDs as well as defective aortic arch artery and pharyngeal arch development, which phenocopies the *Tbx1* null phenotype (59;60). Inactivation of *Fgf8* by *Nkx2.5*, *Mesp1*, *Isl1* and *Mef2c* Cre lines have helped clarify its role in pharyngeal arch artery and OFT development (18;61). Deletion of *Fgf8* in cardiac mesoderm progenitors resulted in severe right ventricular and OFT hypoplasia as well as OFT alignment and septation defects. When *Fgf8* was ablated in the pharyngeal mesoderm at later stages, mutant embryos showed OFT misalignement defects and decreased expression of *Isl1*, *Bmp4* and *Wnt11* in the pharyngeal mesoderm and OFT myocardium (61). These observations demonstrate the importance of Fg8 in the survival and proliferation of the SHF. Generation of *Fgf8/Fgf10* compound mutants further revealed severe OFT and pharyngeal artery defects, demonstrating that the maintenance of proliferation in the SHF is sensitive to FGF dosage (62).

1.1.2.2.3. BMP signaling in recruitment of SHF cells to heart tube

As described in section 1.1.1.2, Bmp signalling is important in the endoderm to specify cardiac mesoderm cells. In 2001, when the SHF was identified, Waldo *et al* demonstrated that blocking Bmp signalling inhibits SHF differentiation and they identified Bmp2 as a candidate molecule in recruiting SHF cells to the arterial pole of the heart (40). Consistent with this hypothesis, overexpression of *Bmp2* in the *Nkx2.5* mutant embryos leads to progenitor over-specification, with subsequent failure in SHF proliferation and OFT truncation defects (63). Thus, *Nkx2.5* regulates SHF proliferation through repression of *Bmp2/Smad1* signalling. Furthermore, conditional inactivation of the Bmp receptor *Bmpr1* by $Isl1^{Cre}$ resulted in RV hypoplasia, defective OFT septation, underdeveloped valves as well as VSDs and ASDs (64). These observations reveal that Bmp signalling is required for right ventricular and OFT morphology

In the mouse, *Bmp4* is expressed in the OFT myocardium as well as the splanchnic and branchial arch mesoderm and deleting *Bmp4* in the cardiac mesoderm results in defective OFT septation and abnormal morphogenesis of the branchial arch

arteries (65). Furthermore, mouse embryos lacking both *Bmp4* and *Bmp7* display abnormal OFT elongation, suggesting that ligand redundancy may mask an essential role for Bmp signalling in recruitment of SHF cells. More recently, *Bmp4* inactivation in the SHF revealed a role for Bmp4 in OFT endocardial cushion remodelling and mutant embryos displayed abnormal morphology of semilunar valves, VSDs, PTAs and defective cushion remodelling (66). Moreover, *Bmp2* and *Bmp4* were shown to interact in multiple aspects of cardiac development, including OFT elongation, proper positioning of the outflow vessels and in septation of the atria (67;68). Altogether, these observations reveal that Bmp2 and Bmp4, expressed by the OFT myocardium, are important for inducing differentiation of SHF progenitors at the arterial pole.

1.1.2.2.4. Hedgehog signaling in survival of SHF

In addition to the FGF and BMP pathways, Hedgehog (Hh) signalling has been implicated in the SHF survival and deployment. Mouse embryos with a null mutation in *Shh* have defective arch artery and OFT patterning defects, as well as abnormal development of neural crest (NC) cells, leading to the formation of a single outflow vessel (69). Recently, conditional deletion of *Shh* by $Nkx2.5^{Cre}$ revealed that Hh signaling is required for the survival of the pharyngeal endoderm, which impacts on both the SHF and neural crest cells (70). Shh signalling is required in NC cells for their survival, in the SHF for OFT septation and finally, in the pharyngeal endoderm for survival and also to provide signals to the SHF for OFT lengthening. Embryos lacking the Hh receptor smoothened (*smo*) have a very severe cardiac phenotype and fail to develop beyond the heart tube stage; *Shh;Ihh* compound mutants display an identical phenotype, revealing functional redundancy between Shh and Indian (Ihh) signalling (71). Recently, deletion of *smo* in the *Isl1* domain resulted in OFT shortening and PTAs, phenocopying the $Shh^{-/-}$ phenotype. Furthermore, the conditional mutant embryos displayed elevated cell death, downregulation of *Tbx1* in the pharyngeal mesoderm and *neuropilin2* in the OFT. Thus, a spatial requirement for Hh signalling within Isl1 expression domains is needed for aortic arch and OFT formation.

1.1.3. Cardiac looping

The early embryonic heart is a single, relatively straight tube. During the early phases of vertebrate cardiac development, the primitive heart tube bends and twists (loops) to the right, a process referred to as cardiac looping (Figure 1.1). This process

not only establishes the right-left asymmetry of the ventricular chambers but is also important to bring the segments of the heart tube and vessels into the correct conformation for chamber specification and septation to create two parallel circulations. Cardiac looping is therefore regarded as a key step in cardiac morphogenesis and slight perturbations in the looping process can result in congenital cardiac malformations. Cardiac looping involves four different processes: formation of a C-shaped loop, elongation to form the S-shaped loop, convergence of the inflow and outflow poles, and rotation and wedging of the aorta between the atrioventricular valves (72). However, the mechanisms that control C-looping and S-looping morphogenesis remain poorly understood.

1.1.3.1. C-shaped loop

Formation of the C-shaped loop involves two independent steps: ventral bending and rotation to the right side (73;74). During dextral looping (or C-looping), the primitive straight heart tube bends ventrally to form a C-shaped tube. There is a coordinated rightward rotation that displaces the ventral surface of the heart tube on the outer surface of the C-shaped loop, resulting in positioning of the heart to the right of the midline. At the end of dextral-looping, the embryonic ventricles are still in a primitive position cranial to the venous pole of the heart (75).

Twisting or rotational movements of the heart tube during looping are influenced by morphogenetic forces that arise within the heart tube or that are extrinsic to the heart (73;76-78). In fact, destabilization of the actin cytoskeleton, by using agents that inhibit actin polymerization, disrupts ventral bending (73). Moreover, when the splanchnopleure is removed from the embryonic chick heart, torsion of the heart tube is suppressed (79).

1.1.3.2. S-shaped loop and convergence

After bending and rightward looping have been initiated, morphological changes occur, which are characterized by: 1) reduction in the distance between the foregut and the outflow and inflow attachments points and 2) caudal displacement of the ventral bend, which was initially cranial to the inflow attachment and atrium (74). During early S-looping, the embryonic ventricles are displaced caudally to the future atrial chambers, thus adopting their final position (75). At the end of early S-looping, the proximal part of the OFT is located to the right of the common atrium and atrioventricular (AV) canal

whereas the AV canal lies to the left of the body midline. In the late phase of S-looping, the heart loop untwists, causing a ventral shift in the right ventricle whereas the proximal part of the OFT undergoes a ventral and leftward shift and the AV canal a rightward shift.

During this final stage of looping, the outflow and inflow poles converge as the last segment of the OFT and chamber identity becomes established (74;80). A major factor in formation of the S-shaped loop and convergence appears to be the addition of cells from the SHF to the arterial pole. Failure of addition of these cells to the arterial pole disrupts the morphology of the S-shaped loop, creating a shortened heart tube and preventing caudal displacement of the OFT, resulting in failure of convergence. The consequence is abnormal cardiac development, which ranges from the absence of the right ventricle and conus to conotruncal defects such as Tetralogy of Fallot or double outlet right ventricle.

1.1.3.3. Wedging

Wedging corresponds to the movement of the aorta behind (or caudal) the pulmonary trunk and occurs during septation of the cardiac chambers. Wedging is dependent on retraction and rotation of the truncal myocardium by about 45° (81). One mechanism that has been proposed for the elimination of myocytes in the outflow myocardium is apoptosis. This is supported by the fact that treatment of chick embryos with caspase inhibitors prevents normal cell death and leads to a failure in OFT shortening and double outlet right ventricle (82;83). When wedging of the aorta fails to happen, the aorta gets either shifted over the ventricular septum and right ventricle, a configuration known as overriding aorta, or settles to the right of normal position (84;85). These defects are observed in conotruncal defects such as Tetralogy of Fallot or double outlet right ventricle.

1.1.4. Establishment of left-right asymmetry

Left-right (L-R) patterning plays an essential role in creating asymmetry in the cardiovascular system. The heart tube is initially linear and symmetric along the left-right axis but becomes divided into distinct regions along the anterioposterior axis, which will give rise to the atria and ventricles. This bilateral symmetry is broken down by rightward looping of the heart, which is the earliest macroscopic sign of L-R patterning. The first indication of cardiac L-R asymmetry is observed right after

gastrulation, once the cardiac cells are residents of the primary heart fields (86). This is consistent with the observation that L-R determination in the *Xenopus* heart occurs when the primordial is a simple sheet of mesoderm (87). A major breakthrough in the understanding of L-R patterning occurred in 1995 when Levin *et al* discovered that *sonic hedgehog*, *nodal* and *activin* can function as L-R asymmetry genes in the chick (88). Since then, the field of L-R asymmetry has grown rapidly and several genes have been identified that act in concert to establish L-R patterning.

So far, the left-sided expression of *Nodal* has been shown to be essential for left-right development and is well conserved among vertebrates (88-90). In *Xenopus* and chick, ectopic nodal expression alters cardiac laterality (91;92). The definitive role of *nodal* in mouse embryos has been lacking because null mutation blocks gastrulation. However, heterozygous *nodal* mice show an absence of *pitx2* expression and left-right organ defects (93). Furthermore, when nodal expression was reduced specifically in the node, loss of asymmetric *nodal* and *pitx2* expression in the lateral plate mesoderm was observed as well as defects in organ left-right orientation (94;95). Similarly, knocking down *nodal* expression in zebrafish using antisense morpholino oligonucleotides prevents asymmetric *pitx2* expression and alters L-R asymmetry in the heart (96). Overall, these studies indicate a requirement for nodal in normal cardiac left-right morphogenesis, through activation of the target gene *pitx2*.

In addition to nodal, *lefty-1* and *lefty-2*, which belong to the TGFβ family, are involved in the relay of L-R patterning. In the mouse, *lefty-1* is predominantly expressed in the left prospective floor plate whereas *lefty-2* is more expressed in the left lateral plate mesoderm (97;98). Although lefty and nodal share the property of being expressed on the left side of the embryo, they seem to have different functions regarding L-R development. Whereas nodal promotes leftness, lefty appears to inhibit this process. Of note, targeted deletion of *lefty-1* in mouse embryos causes bilateral expression of *nodal*, *pitx2* and *lefty-2* in the lateral plate mesoderm and perturbed organ left-right orientation (97). Moreover, chick, zebrafish, mouse and Xenopus lefty homologs are able to antagonize nodal signaling (98). Thus, this suggests that lefty-1 in the midline is presumed to provide a barrier required to prevent the spread of left-side nodal to the other side of the embryo.

A major downstream effector of the nodal pathway in cardiac development is the *Pitx2* homeobox gene (99;100). *Nodal* expression is transient whereas *Pitx2* expression is maintained during formation of handed organs such as the heart and gut (99;101). Thus, it seems like Pitx2 is the molecular transducer of embryonic L-R signalling. Three Pitx2 isoforms (*Pitx2a*, *Pitx2b* and *Pitx2c*) are expressed throughout development and only *Pitx2c* is expressed asymmetrically within the LPM and developing heart (102). Initial studies revealed that right sided overexpression of *Pitx2c* in chick and *Xenopus* causes reversed cardiac looping (99). These findings, coupled with the observations that *Pitx2c* is expressed by other organs undergoing looping, suggested that Pitx2c is involved in cardiac looping morphogenesis (99). However, *Pitx2abc* triple mutants and *Pitx2c* null mice do not exhibit reversed cardiac looping, indicating that Pitx2c is dispensable for this aspect of the looping process in mice (103;104). Nonetheless, in the *Pitx2c* null embryos displayed numerous cardiovascular defects including complete AV canal defects, DORVs, transposition of the great arteries and right ventricular hypoplasia, suggesting important functions in cardiac and vascular morphogenesis (104;105). Moreover, Pitx2c loss of function in *Xenopus* causes abnormal shifting/rotation of the OFT, which are causatively linked with DORV, TGA and VSD. Thus, the abnormal OFT looping that is associated with impaired Pitx2c function could account for these defects.

On the right side of the node, several signalling pathways are required to repress right-sided nodal expression and for the induction of *cSnr*, a transcription factor that is normally restricted to the right LPM (106). In response to Activin, BMP4 is induced on the right side of the LPM and in turn induces the expression of FGF8 and PCL2, the latter functioning to repress right-sided expression of sonic hedgehog (107;108). A role for BMP4 in mouse L-R patterning came from the observation that in the *BMP4* null embryos, nodal expression is severely reduced in the node and absent from the LPM (109). In addition, tetraploid rescued BMP4 mutant embryos show no evidence of heart looping and have reduced expression of nodal in the node and LPM, suggesting that BMP4 signalling is essential to establish left sideness (109). Lack of bilateral expression of *nodal*, *lefty2* and *pitx2c* in *smad5* null embryos further support the notion that upstream BMP signalling is required for repression of nodal activity in the right LPM (110).

1.1.5. Chamber formation and maturation

As the heart tube loops, the cardiac chambers form by expansion of portions of the linear tube. The myocytes of the linear heart tube are in a low proliferative state. Then, at specific regions in the tube, myocytes will increase in size and will reinitiate cell division (111). Under the control of the chamber-specific gene program, these cells will acquire the properties of chamber myocardium (112). Other portions of the linear tube, including the OFT, AV canal and inflow tract, will retain their primary myocardial phenotype and are interconnected to one another at the inner curvature. At the caudal portion of the tube, the primary myocardium differentiates into chamber myocardium of the left and right atrial appendages. Concomitant with chamber formation, typical trabeculations develop, which are more pronounced in the ventricles than in the atria. All of these processes, growth, proliferation and differentiation, cause the apical parts of the ventricles and the atrial appendages to expand locally. This configuration now allows the systemic and pulmonary circulations to be properly arranged in parallel.

1.1.5.1. Transcriptional regulation of chamber development

Chamber specification occurs through distinct transcriptional networks that govern the identity of the myocardium. Ventricular chamber specification occurs at distinct zones at the outer curvature of the looping heart tube, which is marked by expression of *Hand1*, *Cited1*, *Irx1/2/3*, *Cx40* and *Cx43*, *Nppa* and the cytoskeletal protein *Chisel* (72;113).

The basic helix-loop-helix transcription factors *Hand2* and *Hand1* are coexpressed in the developing heart tube but become restricted to the right (Hand2) and left (Hand1) ventricles respectively. *Hand1*$^{-/-}$ embryos die between E8.5-E9.5 due to yolk sac abnormalities and heart development does not progress beyond the looping stage (114). However, conditional deletion of *Hand1* in the heart resulted in left ventricular hypoplasia as well as hyperplastic AV valves (115). In the *Hand2*-null embryos, the right ventricle progenitor cells undergo massive apoptosis, impairing expansion of this segment, thus resulting in the absence of the right ventricle (116). Furthermore, generation of *Hand1/Hand2* compound mutants revealed that ventricle morphogenesis is sensitive to the dosage of the Hand genes (115).

The transcription factors *Nkx2.5*, *Gata4* and *Tbx5* have been implicated in induction of cardiac chamber development based on their expression pattern early in

cardiac development and on the complete/conditional knockout phenotypes (117). To summarize, *Nkx2.5* null mouse embryos form a beating heart tube but looping does not occur and subsequently, the cardiac chambers fail to form (118). Moreover, expression of several genes involved in myocardial differentiation, including *Nppa*, *Nppb*, *MLC2V*, *N-myc*, *MEF2C*, *Hand1* and *Msx2*, was disturbed in the mutants, suggesting a requirement for Nkx2.5 in chamber specification. A null mutation in *Tbx5* leads to embryonic death by E10.5, cardiac looping does not take place and chamber specific genes *Cx40* and *Nppa* are absent or strongly reduced (119). Moreover, misexpression of *Tbx5* in the heart tube inhibits IVS formation and expression of *Hand1* and *Hand2* was changed concomitant with the cardiac anomalies (120;121). Thus, chamber development seems to be sensitive to Tbx5 dosage. Lastly, *Gata4* null mice die early in embryogenesis due to defects in extraembryonic endoderm (34;35). A number of conditional knockout strategies have shown that loss of *Gata4* leads to ventricular hypoplasia and myocardial thinning, suggesting a function in cardiomyocyte proliferation as well as ventricular morphogenesis (122;123).

Tbx5 has been shown to cooperate with Nkx2.5 to activate expression of several genes including *Nppa* and *Cx40* (119;124). On the other hand, Tbx2 is able to form repressive complexes on the *Nppa* promoter and competes with Tbx5 for binding to the T-box binding sites. Thus, the formation of positive and negative complexes can provide a potential mechanism to generate chamber-specific gene expression (125). *Nppa* is initially expressed throughout the chamber myocardium but becomes restricted to the atrial myocardium as development proceeds. Restriction of *Nppa* gene expression to the atrial myocardium is regulated by combinatorial interactions between Nkx2.5, Gata4, Tbx5 and SRF. Efficient expression of *Nppa* does not necessarily require the GATA and NKE binding sites but combinatorial interaction between these two factors is essential in restricting *Nppa* expression to the atrium (126). When the NKE site and the proximal GATA binding element were mutated, ectopic *Nppa* expression was observed in the ventricles and OFT, in addition to tissues outside the heart (126). The importance of genetic interactions between these factors is underscored by the presence of mutations that lead to congenital heart malformations. Interestingly, the mutation G295S in *Gata4* was demonstrated to abolish physical interactions with Tbx5, thus leading to ASDs (127). Similarly, two *Tbx5* missense

mutants, which have lost the ability to bind DNA and to interact with Nkx2.5, lead to ASDs (128-130).

In addition, formation of chamber myocardium needs to be prevented at the AV canal, inflow tract, OFT and inner curvature, which is accomplished by the transcription factors *Tbx2* and *Tbx3*. *Tbx2* and *Tbx3* are expressed in the inflow tract, AV canal and OFT during early cardiac development (125;131). *Tbx2* null embryos show ectopic expression of chamber genes including *Nppa*, *Cx40* and *Cx43* in the AV canal whereas overexpression of *Tbx2* early in development leads to absence of *Nppa* and *Cx40* expression and prevents chamber formation (43;125;132). These observations clearly show that Tbx2 is required to suppress chamber formation in the AV canal. Bmp2 induces expression of *Tbx2* in the AV canal and Tbx2 expression is abolished in the AV canal of *Bmp2*$^{-/-}$ mice, leading to up-regulation of *Cx40*, *Nppa* and *Chisel* (133;134). Moreover, application of Bmp2 soaked beads to the mesoderm of chick embryos leads to induction of Tbx2 (135). Bmp2 is also able to induce expression of *Nkx2.5* and *Msx2*, which can physically interact and cooperate with Tbx2 and Tbx3 in regulation of chamber gene expression (131;136). Eventhough *Msx2*$^{-/-}$ mice do not display AV canal defects, *Msx1/Msx2* compound mutants show endocardial cushion defects associated with aberrant expression of *Nppa*, *Tbx2*, *Hand1* and *Hand2* (137). Recent studies have shown that mice lacking *Tbx3* display ectopic expression of *Cx40* and *Cx43* in the sinus node, whereas the bundle branches fail to develop (138;139).

In order to allow proper chamber formation, expression of Tbx2 and Tbx3 must be tightly regulated and confined within the AV canal and this is done by another member of the T-box family of transcription factors, Tbx20. In the heart of *Tbx20*$^{-/-}$ embryos, chamber formation does not occur, as evidenced by the loss of Nppa and Cx40 expression in mutant heart tube (140-142). Furthermore, expression of Tbx2 was observed in the cardiac crescent and throughout the linear heart tube in *Tbx20* null embryos, showing that Tbx20 is a repressor of Tbx2. Recently, Singh *et al* demonstrated that *Tbx20* is able to repress smad1/smad5 activity, between E7.5-8.5, in order to delay and restrict activation of Tbx2 in the AV canal (143). Thus, restricted induction of *Tbx2* by Bmp signalling in the OFT and AV canal may underlie the inhibition of chamber myocardial genes and maintenance of the primary phenotype in these regions.

1.1.5.2. Formation and patterning of the trabeculated myocardium

In both mouse and human, trabeculations start to form after looping of the linear heart tube, concomitant with expansion of the ventricular chambers (Figure 1.1). The trabeculae can comprise up to 80% of the cardiac mass and generate much of the contractile force of the heart, from E9.5 to E14.5, as well as rapidly propagate the electrical impulse throughout the ventricles. Prior to septation, the trabeculae help in maintaining separate blood flow through the embryonic ventricular chambers and also contribute extensively to the development of the ventricular conduction system (section 1.1.6). Several genes including *Hand1*, *Cited1*, *Irx5*, *Nppa*, *Cx43* and *Chisel*, are expressed in the trabecular zone (144).

Signalling from the endocardium is essential for trabecular growth. Neuregulin-1 is secreted by the endocardium and signals to its tyrosine kinase receptors ErbB2 and ErbB4, which are expressed in the myocardium. Targeted deletion of *neuregulin-1* (*NRG1*) in mice leads to embryonic death at E10.5 due to aberrant cardiac development. The trabeculea fail to form properly, resulting in an enlarged common ventricle, reduced blood flow and arrhythmias (145). *erbB2* and *erbB4* deficient embryos display a very similar phenotype, with fetal death at E10.5 due to lack of trabeculation (146;147). This suggests that NRG1 provides an essential paracrine signal to myocardial cells, through the ErbB2/ErbB4 heterodimer, for trabecular formation. Another key molecule involved in formation of trabecules is Bmp10. In mouse embryos, Bmp10 is expressed transiently but specifically in the trabeculae between E9.0 and E13.5 after which it becomes restricted to the atria. Targeted inactivation of *Bmp10* results in embryonic death at E10.5 and mutant embryos have hypoplastic ventricular walls and form primitive trabeculae (148). These observations suggest that Bmp10 is not required for initial formation of the trabeculae but is rather essential for further trabecular growth by maintaining appropriate proliferation, mainly by repressing the cell cycle regulator $p57^{kip2}$. Recent studies have revealed that Bmp10 possibly acts downstream of the Notch pathway. Consistent with this, *Notch1* or *RBP-Jκ* mutant embryos showed impaired trabeculation as well as reduced proliferation and expression of *EphrinB2*, *NRG1* and *Bmp10*, which are all involved in trabeculae formation (149).

1.1.6. The cardiac conduction system

The cardiac conduction system initiates and coordinates electrical signals that cause the rhythmic and synchronized contractions of the atria and ventricles. In higher vertebrates, this system is composed of the nodes and the ventricular Purkinje fiber network, with the sinoatrial (SA) node being the primary pacemaking component that generates the electrical impulse (150). In a mature heart, the SA node is located at the junction of the superior canal vein and right atrium. Following initiation of a cardiac action potential within the SA node, the electrical impulse propagates rapidly through the fast-conducting atrial muscle to initiate contraction of the atria. The impulse then spreads slowly through the atrioventricular node (AV node), which is located at the base of the atrial septum adjacent to the tricuspid valve. This slow conducting node forms the only route from the atria to the ventricles. The main function of the AV node is to separate, and to some extent insulate, the activation of the atrial chambers from that of the ventricles. After this slight delay, the electrical impulse travels through a fast conducting His bundle (AV bundle) and its bundle branches that are located on both sides of the ventricular septum. The bundle branches divide on either side of the ventricular septum into a highly ramified network of Purkinje fibers, which rapidly transmits the impulse to the ventricular working myocardium from the apex to the base.

Higher vertebrates will develop a SA node in the sinus venosus, an AV node within the AV canal and a fast ventricular conduction network to activate both ventricles efficiently and co-ordinately. These different components originate from myocardial cells as evidenced by lineage tracing studies of the early avian (151;152). Furthermore, these studies also demonstrated that labelled neural crest or proepicardial cells do not contribute to the conduction system components. The cardiac conduction system is innervated by cardiac ganglia that are derived mostly from neural crest cells (153). In addition, a large number of non-cardiac cells such as fibroblasts can be detected in the mature conduction system and these are derived from the epicardium, endocardium and neural crest (153-155). Eventhough the nerves and fibrous tissues are required for the formation and function of the conduction system, the cardiomyocytes are essential for the generation and propagation of the action potential.

1.1.6.1. Sinus node development

The mature SA node consists of an elongated structure with a head that is wrapped around the superior canal vein, including the border of the right atrium, and a tail along the terminal crest (156;157). In the early embryonic heart, all cardiomyocytes display pacemaker activity but the cells at the venous pole have the highest intrinsic rate and thus function as the predominant pacemaker (155;158).

Tremendous progress has been made over the years to increase our understanding of the molecular pathways involved in SA node formation. In higher vertebrates, it is thought that the SA node develops from primitive myocardium located in the venous pole (inflow region) of the embryonic heart (155;158). Until E9.0-9.5, *Nkx2.5* is expressed in all primitive myocardial cells derived from the primary and secondary heart fields whereas mesenchymal cells at the caudal-ventral side of the inflow tract express the T-box transcription factor *Tbx18* (156;159). Concomitant with heart tube elongation and formation of the four chambered heart, *Tbx18* expressing mesenchymal cells are added to the tube to form the sinus horns, which will eventually differentiate into the SA node around E10-11 (156;159). Interestingly, the myocardium of the sinus venosus and SA node expresses *Tbx18* but not *Nkx2.5*, suggesting these components of the conduction system differentiate from a $Tbx18^{+}$/Nkx2.5-negative mesenchymal precursor population. Consistent with this, $Tbx18^{+}$ non-cardiac precursor cells were able to differentiate into an $Hcn4^{+}/Nkx2.5^{-}$ pacemaker myocardium in culture (156). Moreover, *Tbx18* null mice fail to form the sinus horns, demonstrating that Tbx18 is required for the recruitment of these mesenchymal progenitors to the cardiac lineage at the venous pole (156;159).

The hyperpolarization-gated cyclic nucleotide cation-activated channel *Hcn4*, which is essential for pacemaker activity of the SA node, is expressed at E8.0 in the venous pole of the linear heart tube and later becomes confined to the sinus horns and eventually to the SA node region by E12 (157;160;161). Interestingly, *Hcn4* null mice die at E11.5 due to strong reduction in I_f current resulting in lack of formation of a mature pacemaker while its overexpression mimics pacemaker properties in cell culture (157;160;162;163). Heterozygous mutations in the *Hcn4* gene were found in human with brachycardia (164). Expression of *Hcn4* initially overlaps with that of *Nkx2.5* in the linear heart tube but subsequently becomes downregulated in this domain and

activated in Tbx18+/Nkx2.5-negative myocardial cells of the sinus horns. Thus, expression of *Hcn4* gets shifted into the newly added cardiac cells at the venous pole (sinus venosus). This highly suggests that *Hnc4* is repressed in the *Nkx2.5*-positive myocardium that is fated to form the atria and atrial layer of the semilunar valves (159). Consistent with this, *Nkx2.5* deficient embryos show ectopic expression of *Hcn4* and *Tbx3* in the heart tube and fail to induce *Cx40* in the atria, suggesting that Nkx2.5 negatively regulate Hcn4 and Tbx3 (165). These observations also indicate that Nkx2.5 is required to establish a boundary between the atria and SA node in order to prevent the SA node phenotype from invading the atria and vice-versa. *Tbx3* has emerged as a critical regulator of the cardiac conduction system, especially in regulating the function of the SA node (138;166). Tbx3 is expressed in the SA node, the AV node, the AV bundles and proximal bundle branches during cardiac development (166). Importantly, *Tbx3* null embryos show expansion of working atrial gene expression including *Cx40*, *Cx43*, *ANF* and *Scn5a* into the SA node domain (138). Moreover, ectopic expression of *Tbx3* within the atria is sufficient to activate ectopic expression of pacemaker genes including *Hcn4*, *Cx30.2* and *Cav3.1* while repressing the atrial phenotype (138). Thus, *Tbx3* is required for the induction and maintenance of the SA node gene program while preventing the expansion of atrial gene expression.

1.1.6.2. AV canal specification and AV node formation

The second major site in the heart with pacemaker potential activity is the AV node. The linear heart tube consists of primitive myocardium that displays poor contraction and slow conduction of the electrical impulse (155). During early cardiac development, the linear heart will elongate by addition of cells at both poles and differentiate into atrial and ventricular chambers as the tube loops. Chamber myocardium goes on to acquire high conductance gap junctions, including Cx40 and Cx43, as well as fast conduction and rapid contraction and finally pronounced trabeculae in the ventricular chambers. In contrast, the myocardium of the sinus horns, the AV canal, the inner curvature and OFT preserve their original embryonic phenotype. It is generally thought that the AV node as well as the AV bundles, AV valves and lower rim of the atrium originate from the AV canal (167). The molecular mechanism underlying AV canal formation and differentiation has been extensively studied, which is nicely reviewed in section 1.1.5.1.

The transcription factors Nkx2.5 and Tbx5 are also expressed in the AV canal, where they play important roles. Of note, *Nkx2.5* haploinsufficiency leads to atrial septal defects and AV conduction abnormalities during postnatal life (168;169). In addition, *Nkx2.5*$^{+/-}$ mice display strongly hypoplastic AV node (170). Lastly, neonatal mice with ventricular specific deletion of Nkx2.5 display first-degree AV block which progresses to high grade AV block later in life (171). Similarly, haploinsufficiency of *Tbx5*, which is a model of Holt-Oram syndrome, is characterized by failure of AV canal maturation, patterning defects of the left and right bundle branches and right bundle branch block (172). Adult *Tbx5*$^{+/-}$ mice exhibit various degrees of conduction defects including AV block and SA node dysfunction and 50% of these animals have second degree AV block (119).

During embryonic development, the AV canal retains the slow conduction property of the primitive myocardium, thus functioning as an AV node equivalent. The action potential can be propagated from the atria to the ventricles and back through the ring of AV myocardium that connects these chambers (173;174). Soon after septation is completed, connective tissue from the AV cushion and epicardial mesenchyme will invade the myocardium to form the annulus fibrosus, which acts to physically separate and insulate the atria and ventricles. The only connection that remains is the AV bundle, which connects the AV node at the atrial side with the ventricles. However, the mechanism that underlies formation and morphogenesis of the AV node from the embryonic AV canal is not well understood.

1.1.6.3. The ventricular conduction system

In the mature heart, the ventricular conduction system consists of the AV bundle (His bundle), the bundle branches and the Purkinje fiber network. This system acts to transmit the electrical impulse rapidly from the AV bundle and proximal bundle branches to the ventricular working myocardium starting at the apex. In the mouse, the gap junction *Cx40* is essential for the propagation of the fast electrical impulse through the AV bundle and bundle branches (175;176). It also serves as a specific marker able to distinguish the fast-conducting components (bundle branches and Purkinje fibers) from the working myocardium of the ventricles (177-179). A number of hypotheses have been made about the origin of the AV bundle but it is now believed to originate from the crest of the ventricular septum (180;181). In the mouse, AV bundle cells can

be first observed around E10-11 at the top of the forming ventricular septum. With further development of the ventricular septum, left and right bundle branches develop from the subendocardial myocytes and bifurcate from the His bundle. AV bundle development is sensitive to haploinsufficiency of *Tbx5*, *Nkx2.5* and *Id2* or combinations of them as well as loss of *Tbx3* (139;182). Thus, these findings suggest that an Nkx2.5-Tbx5-Id2 dependent pathway is necessary for the formation of the AV bundles.

The embryonic heart of higher vertebrates display fast conduction well before septation is completed, at the time when trabecules have just emerged (183;184). These observations prompted researchers to suggest that the trabecules are the structural and functional precursors of the Purkinje fibers. In the chicken and mouse, *Cx40* is initially expressed in the atria and ventricle primordia but as development proceeds, becomes more dynamic in the ventricles with expression confined to the trabecular network and subsequently to the ventricular conduction system, concomitant with the formation of the compact layer at the epicardial side (177). Soon after birth, a further maturation step that is dependent on Nkx2.5 and epicardial cells takes place, remodelling the trabecular zone into the Purkinje fibers that are only one to a few cells thick (185;186).

1.1.7. Formation of the cardiac valves

The complexity of the vertebrate heart increased by the progressive colonization of land by vertebrates and allowed adaptation to a new environment. The main consequence of this dramatic change was a division of the bloodstream into two separate circuits, pulmonary and systemic. This physiological change led to a spatial reorganization of the ancestral fish heart into a four chambered organ that is composed of two atria and two ventricles. In order to allow coordination and efficient blood supply to the lungs and the rest of the body, the heart of higher vertebrates developed a very sophisticated valve system. The cardiac valves are fibrous structures that originate mainly from the endocardium; they are not completely functional until late gestation and are fully mature only after birth.

The cardiac valves of higher vertebrates can be classified as atrioventricular (AV) and semilunar (OFT) valves (Figure 1.3). The AV valves originate from the AV cushions and separate the atria from the ventricles. The mitral valve controls blood flow between the left ventricle and left atrium whereas the tricuspid valve regulates blood

blow between the right atrium and right ventricle. The leaflets of the AV valves (two in the mitral and three in the tricuspid) are flattened laminar structures that are attached to the annulus fibrosus at their base. The tip of the valve is attached to tendinous chords that provide support to the leaflet. The tendinous chords are in turn supported by papillary muscles, which are thick expansions of the myocardium and together, they constitute the tension apparatus. The semilunar valves are made of three leaflets and originate from endocardial-derived and neural-crest derived mesenchyme. The aortic valve controls blood flow between the left ventricle and aorta whereas the pulmonary valve regulates blood flow between the right ventricle and the pulmonary artery.

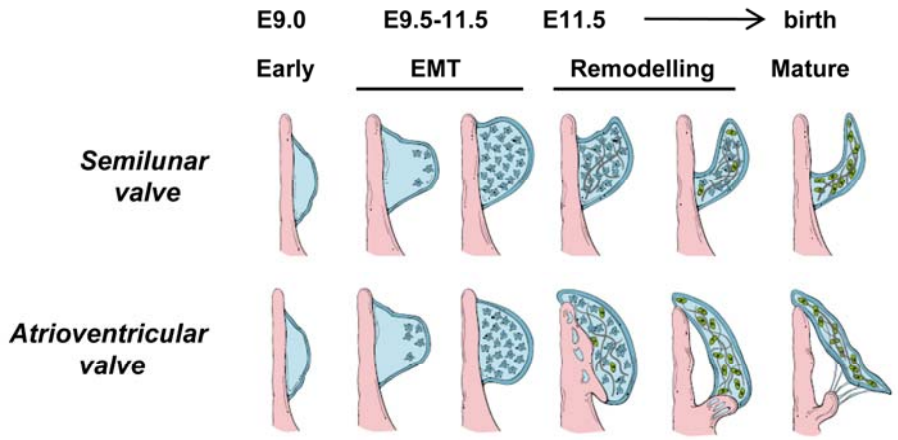


Figure 1. 3. Development of the semilunar and atrioventricular valves

EMT: epithelial-mesenchymal-transition. Adapted from Camenisch T *et al*., *Cardiac development and regeneration*, 2010: 363-387.

1.1.7.1. The extracellular matrix

The first evidence of endocardial cushion formation is the appearance of swelling in the AVC and OFT regions of the looping heart around E9.5 in the mouse, E3 in chick and E31-35 in human (Figure 1.1). Cushion formation is induced by signals emanating from the myocardium lining the cushion regions, where expression of chamber specific genes in the AVC and OFT is inhibited and synthesis of ECM components increased. This increased ECM or ‘’cardiac jelly’’ deposition between the myocardium and endocardium causes the tissue to protrude or swell into the interior lumen of the heart forming the endocardial cushions (187). Even at this early stage, the endocardial cushions are able to act as a barrier to prevent the backflow of blood

through the heart tube. Signals emanating from both the myocardium and endocardium of the AVC and OFT are necessary for proper endocardial cushion formation and epithelial-to-mesenchymal transformation (EMT).

The ECM of the cardiac cushions is a unique and dynamic mixture of proteins, proteoglycans and glycosaminoglycans and is devoid of cells prior to EMT. Major components of the ECM include hyaluronan (HA), the proteoglycans aggregan and versican, and glycoproteins like laminin, collagens, fibronectin, fibulins, fibrillins and periostin (188-191). Importantly, the formation and expansion of the cardiac jelly precedes the epithelial-to-mesenchymal transformation and production and migration of mesenchymal cells. The importance of the cardiac jelly in endocardial cushion development is highlighted in the Trisomy 16 (Ts16) mouse model. In Ts16 embryonic hearts, which is an accepted model for human Trisomy 21, the cushion volume is greatly elevated due to increased cardiac jelly content but attenuated mesenchyme production. These embryos have a very high incidence of AV septal defects as a result of abnormal cushion formation, suggesting that the proper formation of the ECM within the cushions is important both for valve and septal development. The regulatory mechanisms that govern the production of the cushion ECM are not well understood but some insights have been acquired from mouse genetics. Targeted inactivation of either hyaluronan synthase 2 (*Has2*) or versican in mouse embryos results in embryonic lethality from heart defects including disrupted cushion morphogenesis (192-194). Moreover, the zebrafish *Jekyll* mutants exhibit hypoplastic cushions similar to mice lacking *Has2* (195). *Jekyll* mutants lack a functional uridine 5'-diphosphate(UDP)-glucose dehydrogenase gene that is required for the biosynthesis of HA, likely suggesting that the defects of the *Jekyll* mutants are related to deficits in HA production and activity. Further studies with the *Has2*-null embryos showed that the epithelial-mesenchymal transition was inhibited in *Has2*-deficient AV explants (192). This defect was however rescued with exogenous HA, or by expressing *Has2* cDNA, to *Has2*$^{-/-}$ cushion explants cultures.

1.1.7.2. Epithelial-to-mesenchymal transformation

Cardiac cushions are the primordial of the valves and septa of the adult heart and play a crucial role in maintaining anterograde blood flow in the embryonic heart. Beginning at E9.5, the cardiac cushions of the AV and OFT regions begin to be

populated by mesenchymal cells by a process known as epithelial-to-mesenchymal transformation (EMT). EMT is a finely regulated mechanism that describes a series of events during which epithelial cells lose many of their epithelial characteristics through complex changes in cell architecture and behaviour, and take on properties that are typical of mesenchymal cells (196). Most of the early insights into the process of endocardial EMT came from studies of a three-dimensional collagen gel culture system (197). In this system, the AV and OFT regions are dissected and isolated before the onset of EMT and are explanted onto the surface of the collagen gel, after which a subset of endocardial cells will transform into mesenchymal cells and invade the collagen matrix. This technique allows for the quantification of EMT by determining the subsequent invasion of the collagen gel by endocardially derived cells and has been instrumental in defining the molecular regulation and major steps involved in EMT. The steps in EMT are (1) activation of endocardial cells by the adjacent myocardium, (2) transformation of the endocardial cell, which lose their cell-to-cell contacts and acquire the ability to move within the endocardial layer and form filopodia and lamellipodia and (3) invasion, where the transforming endocardium migrates from the endocardial cell layer into the cardiac jelly. As development progresses, the mesenchymal cells undergo exhaustive proliferation, resulting in the fusion of the cardiac cushions. Further remodelling of the endocardial cushions results in the formation of thin protruding leaflets comprised of endocardial cells and ECM that go on to form the heart valves. In the AV canal, EMT-derived mesenchymal cells are the sole contributor to the mitral and tricuspid valves, whereas in the OFT, endocardial and neural crest-derived EMT contribute to the formation of the aortic and pulmonary valves. A large number of molecules, transcription factors and signaling pathways have been implicated in EMT and cushion morphogenesis, including TGFβ/BMP, VEGF, ErbB, NF1/Ras, NFATc1, Notch, Wnt/β-catenin, Twist1, Sox9, Tbx20, Msx1/2 and GATA4 (198-200). A brief description of some of the major players is reviewed in section 1.1.7.4.

1.1.7.3. Heart valve maturation and remodelling

Mature AV valves are derived almost entirely from the endocardial cushions with contribution from the AV myocardium (201;202). Adult human AV valves are composed of three layers of specific ECM proteins: the fibrosa, the spongiosa and the atrialis. The fibrosa, located at the ventricular side, is composed of densely packed collagen fibers and provides strength; the spongiosa, located centrally, consist of glycosaminoglycans,

which provide cushioning; and the atrialis, continuous with the atrial endocardium, is composed of elastic fibers. The most striking difference between the AV and semilunar valves is the presence of a supporting chordea tendineae on the ventricular side of the tricuspid and mitral valves.

Remodelling of the cardiac valves into a fibrous leaflet correctly attached to its supporting apparatus requires coordinated events including proliferation and apoptosis of mesenchymal cells, fusion of the cardiac cushions and tissue maturation. The number of mesenchymal cells in the endocardial cushions increases progressively after the onset of EMT such that the size of the cushion is appropriate to the region in which they are found (AV canal or OFT) (203). After E11.5, mesenchymal cells lining the myocardial protrusion will proliferate to form the lateral AV cushion, which will eventually form the fibrous component of the mural leaflet. Proliferation continues until the endocardial surfaces of the inferior and superior cushions make contact, initiating fusion between the two cushions. In mice, remodelling of the AV cushion results in the formation of mesenchymal leaflets at E14.5 (201;204). After E14.5, the myocardium of the leaflet will disappear by programmed cell death, freeing the fibrous leaflets. However, little is known about the maturation of these immature leaflets into adult stress-resistant valves due to the lack of mouse models with valve defects that are viable past the stage of EMT. A recent study by Kruithof B *et al* led to a model whereby condensation of mesenchymal cells starts at E15.5 at the atrial side of the leaflet and continues up to E18.5 (205). Cellular proliferation contributes to the leaflet elongation until postnatal day 4.5. Rapid growth of the heart by hypertrophy might then elongate the leaflets by physically pulling at the papillary muscle insertion point. The AV valves then loose cell density and ECM remodelling along the AV axis is achieved 1 week after birth, where two different structural regions can be observed, glycosaminoglycans and versican located on the atrial side and densely packed collagen fibers on the ventricular side.

EGFR signalling is involved at later stages of valve formation, more particularly in the valve remodelling process (206). *HB-EGF* null mice have cardiac valve defects, which is consistent with the restricted expression of this molecule in endocardial cells (207;208). Histological analysis of the mutant mice revealed enlarged semilunar valves and abnormal morphology of the AV valves, which likely results from an increase in the number of mesenchymal cells. This led to the hypothesis that HB-EGF might regulate

the extent of mesenchymal cell proliferation during the remodelling phase. This is supported by the fact that EMT occurred normally in these mice, that the rate of apoptosis was unchanged and that excessive proliferation of the mesenchymal cells was observed (208). Interestingly, increased activation of smads, which are intracellular mediators of BMP signalling, was observed in the hyperplastic valves, suggesting that HB-EGF regulates proliferation of the valves through regulation of smads. Another candidate for the HB-EGF signalling is the fibroblast growth factor (FGF) 4 (209). In chick embryos, FGF-4 is expressed in both mesenchymal cells and myocardial cells, while the FGF receptor (FGFR) 2 is located exclusively in the cushion mesenchymal cells. Furthermore, the authors demonstrated that FGF4 is able to induce proliferation of mesenchymal cells during chick early valve leaflet formation both *in vitro* and *in vivo*.

1.1.7.4. Molecular regulation of cardiac cushion and valve development

The development of the heart valves require complex interactions between signalling molecules, transcription factors and structural proteins that regulate cushion formation, proliferation, expansion, differentiation, lineage diversification and leaflet remodelling. The major signalling pathways involved in endocardial cushion formation and proliferation are reviewed below (Table 1.1).

1.1.7.4.1. TGFβ family as mediator of EMT

Members of the transforming growth factor beta (TGFβ) superfamily are key components in endocardial EMT (210;211). TGFβ binding to the type I and II TGFβ receptors initiates a cascade of events that leads to the activation of the smad transcription factors in the nucleus. In the mouse, TGFβ1 is expressed in the endocardium, TGFβ2 in the myocardium and endocardium of the AV and OFT regions and TGFβ3 is localized in the endocardium and mesenchymal cells after the onset of EMT (212;213). The first line of evidence for a role of the TGFβ family in EMT came from studies where chick AV cushions cultured *in vitro* with exogenous TGFβ1 and TGFβ2 induced endocardial EMT (214). In addition, inhibition of chick TGFβ signalling prevented endocardial EMT, indicating that TGFβ is a specific AV EMT inducer. Further studies then showed that TGFβ2 is required for endocardial cell activation whereas TGFβ3 is important for mesenchyme cell invasion (215;216). Although *in vitro* studies support the notion that TGFβ is essential for EMT during endocardial cushion

Gene	Loss of function phenotype	Function	Reference number
Gata4	Hypocellular cushion	EMT/valve remodelling	719
Tbx20	Decreased cushion mesenchyme proliferation	Proliferation/Differentiation	550, 551
Twist 1	Defective cushion proliferation	Proliferation/migration/ECM organization	551
Sox9	Hypoplastic cushions	Proliferation/Differentiation	855, 856
VEGF	Underdevelopped endocardial cushions	Cushion formation	267, 268
NFATc1	Hypoplastic cushions	Cushion cell proliferation/valve ECM remodelling	272, 273
Msx1/Msx2	Hypoplastic cushions	EMT/cushion formation	137
NRG1	Hypoplastic cushions	EMT/cushion formation	145
ErbB3	Hypoplastic cushions	EMT/cushion formation	281
Has2	Disrupted cushion morphogenesis	EMT	192
ALK2	Small endocardial cushions with reduced number of mesenchymal cells	EMT	245
ALK3	Defective AV cushion formation	AV cushion morphogenesis	241
BMP2	Impaired deposition of the ECM at the heart-valve forming region	Specification of the heart valve-inducing region, EMT	133, 134
BMP4	AV septation defects and small AV cushions, defective OFT cushion remodeling, abnormal semilunar valve development	Remodeling and septation of the atrioventricular and semilunar valves	66, 228
Bmp5/Bmp7	Absence of cardiac cushions	Cushion formation	233
Bmp6/Bmp7	Delayed formation of OFT cushions	Cushion formation	234
Notch1	Hypoplastic cushions	EMT/cushion formation	149, 251
RBP-J	Hypoplastic cushions	EMT/cushion formation	252
Hey2	Pulmonary and tricuspid stenosis, mitral valve regurgitation	Valve formation and maturation	255, 256
Hey1/HeyL	Dysplastic AV and pulmonary valves	EMT	257

Table 1. 1. Loss of function phenotypes of genes involved in valve development

formation, neither *TGFβ1*- nor *TGFβ3*-null mice show an apparent cardiac phenotype (217-219). Only *TGFβ2*-null mice have specific cardiac defects in the valves and septa (220;221). Thus, it appears that there is greater potential for functional redundancy of the TGFβ isoforms in mice. Consistent with this, mice null for both *TGFβ2* and *TGFβ3* have a more severe cardiac cushion phenotype than that observed in *TGFβ2*-mutant embryos alone (222).

EMT of endocardial cushions also correlates both spatially and temporally with the expression of bone morphogenetic proteins (BMP). During murine cardiogenesis, BMP2 and BMP4 are expressed in the AV and OFT myocardium, *BMP6* transcripts are localized in the myocardium of the OFT region and endothelial/mesenchymal cells of the AV canal whereas BMP7 is only expressed in the myocardium of the AV and OFT regions (223-225). *BMP2*- and *BMP4*-null mice die before cushion tissue formation, which complicates analysis of the role of BMPs in EMT (226;227). Subsequent studies using *in vitro* collagen gel assay and conditional deletion of BMP2 in the myocardium indicated that BMP2 is essential for AV canal transition into cushion mesenchyme (133;134). Of note, *BMP2*-deficient embryos had less cardiac jelly and insufficient AV cushion formation and had reduced *TGFβ* and *Has2* expression in the heart, suggesting that BMP may be upstream of these EMT regulators. As no OFT defects were observed in *BMP2*-deficient mice, other BMPs, such as BMP4 and BMP7, may be involved in the formation of the OFT cushion tissue. Analysis of mice with cardiac-specific deletion of BMP4 suggested that BMP4 functions in the proliferation and migration of neural crest cells as OFT septation was impaired in these embryos (65). Consistent with a role in septation, myocardial specific inactivation of BMP4 with TnT^{Cre} mice resulted in proper initiation of cushion formation but improper AV cushion septation (228). Conventional inactivation of either BMP5, BMP6 or BMP7 alone does not produce cardiac defects, again suggesting functional redundancy between family members (229-232). However, *BMP5/BMP7* double mutant mice fail to form cardiac cushions whereas *BMP6/BMP7* double mutant embryos have a marked delay in the formation of the OFT cushions, AV canal and chamber morphogenesis (233;234).

Studies investigating the TGFβ receptors provide even more evidence that the TGFβ signalling is a major EMT inductive stimulus in cardiac cushions. Using a blocking peptide against chick TβRII, Brown *et al* demonstrated that this receptor was required for EMT in chick AV canal cushion explants (235). Targeted inactivation of

TβRII from the endothelium in mice resulted in defective remodelling of the cushion after the onset of EMT (236). Further, endocardial explants from TβRII-mutant embryos resulted in defective EMT, similar to the phenotypes obtained with loss of TGFβ2 both *in vitro* and *in vivo*. Similarly, blocking TβRIII signaling prevents EMT while forced expression of TβRIII in the ventricle endothelium results in EMT in regions where it does not normally take place (237). Mouse embryos deficient for ALK2 or ALK3 die before cardiac development (238-240). Myocardial-specific inactivation of *ALK3* in mice results in embryonic lethality due to a defect in septation of the AV region (241). Interestingly, initial steps of endocardial cushion development are normal in both the AV canal and OFT, but cushions fail to fuse after EMT, suggesting that ALK3 is dispensable for EMT. Further insight into the role of ALK3 in valve morphogenesis was obtained from mice with targeted deletion of ALK3 in the myocardium of the AV canal, which showed defective tricuspid and mitral valve morphogenesis and ventricular pre-excitation similar to Ebstein's anomaly in humans (242). Studies of AV explants in chicks have shown that ALK2 is required and sufficient for EMT (243;244). Similarly, endothelial-specific inactivation of *ALK2* resulted in AV valve and septal defects as a result of failure of endocardial cells to transdifferentiate into mesenchyme in the AV canal (245).

1.1.7.4.2. Notch in specification of EMT

The Notch signalling pathway plays critical roles during mammalian cardiac development. In mammals, the Notch family consists of 4 type I transmembrane receptors (Notch1 to 4) and 5 type I transmembrane ligands, Jagged1, Jagged2, Delta-like (Dll)1, Dll3 and Dll4 (246;247). Upon ligand binding, a protease complex containing gamma secretase cleaves the intracellular domain of Notch, which enters the nucleus and regulates gene expression through binding to the transcription factor RBPJκ. Murine *Notch1*, *Notch2*, *Notch4*, *Jagged1* and *Dll4* and the Notch downstream targets *Hey1*, *Hey2* and *HeyL* are all expressed in the AV canal endocardium at the time of EMT (248-250). In *RBPJκ*-null embryos, Notch1 activity is greatly reduced and valve development is severely compromised (251;252). In addition, *RBPJκ*- and *Notch1*-mutant embryos have a collapsed endocardium and lack mesenchymal cells, suggesting defective EMT. Further analysis revealed that the endocardial cells

remained in close association and did not invade the cardiac jelly, as confirmed in the AV canal explants assay with *RBPJκ*- and *Notch1*-mutant embryos.

The Hey transcription factors have also been linked to cardiac development. *Hey2*-null mice have several cardiac abnormalities including VSDs, pulmonary and tricuspid stenosis, mitral regurgitation AV canal defects and cardiac hypertrophy (253-256). Moreover, *Hey1/2* double mutants die at E9.5 due to severe vascular defects similar to those seen in *Jagged1*-null and *Notch1*-null mice (257). In addition, analysis of *Hey2*-deficient and *Hey1/L*-deficient embryos revealed a defect in AV canal EMT (248). Although the EMT process was initiated normally, the migrating cells failed to successfully undergo complete mesenchymal transformation. In *Notch1*-, *Hey2*- and *Hey1/L*-mutant embryos, the EMT defect was accompanied by decreased expression of matrix metalloproteinase 2, which is required for the migration and invasion of mesenchymal cells into the cardiac cushions (248;258).

Further analysis of the *RBPJκ* and *Notch1* mutant embryos indicated that Notch signalling mediates EMT via TGFβ2 signalling. This is supported by the observation that TGFβ2, which is normally expressed in the AV and OFT myocardium, is downregulated in *Notch1*-null embryos (251). Reduced expression of TGFβ2 in turn results in the absence of *Slug*, a close Snail gene family member that acts as a transcriptional repressor in the OFT and AV canal endocardium during the onset of EMT (259;260). In the *Notch1* null embryos, little or no *Snail* expression is observed, resulting in a failure of the endocardial cells to delaminate and cellularize the cardiac cushion (251). Consistent with this, loss of Notch signalling in endothelial cells was associated with loss of Snail expression and inappropriate VE cadherin expression. In endocardial cushions, *Snail* induces EMT through negative regulation of vascular endothelial (VE), resulting in decreased adhesion between endocardial cells and their delamination from the endocardial layer. Similarly, *Slug* is able to repress VE-cadherin and other endothelial markers such as *CD31* and *Tie2* in endothelial cells (261). Overall, these studies led to the model in which high levels of Notch induce high levels of *Dll4* in endocardial cells. This in turn leads to activation of endocardial cells and the production of an endocardial cell-derived signal that induces the production of TGFβ2 in the myocardium lining the cushions. TGFβ2 then signals back to the endocardium to activate Slug/Snail, which ultimately results in the reduction of VE-cadherin expression

and cell adhesiveness. This allows the cells to delaminate from the endocardial layer and migrate into the cardiac jelly.

1.1.7.4.3. Calcium/NFAT/VEGF in cushion formation

Numerous reports have implicated the vascular endothelial growth factor (VEGF) in the activation, proliferation and eventual remodelling of the cushions cells into the valve leaflet. VEGF was initially recognized as a vascular permeability factor and has been implicated in a wider array of processes including vasculogenesis and angiogenesis. Vertebrates have six VEGF genes, with the most abundant and biologically active isoform being VEGF165. VEGF ligands signal through two tyrosine kinase receptors, Flt1/VEGFR1 and Flk1/VEGFR2, which are expressed in the endocardium (262;263). VEGF binding to Flk1 starts a series of events that leads to the activation of different intracellular signalling pathways, allowing VEGF to participate in many biological processes (262;264).

Many studies have linked VEGF to endocardial cushion formation based on its expression pattern in the heart. Early in cardiac development, VEGF protein is found in most endocardial cells of the primitive heart tube but at E9.5-10.5, its expression becomes more restricted to the AV canal and OFT and also to the myocardial cells underlying the cardiac cushions (265;266). What is most striking about VEGF it that while it is necessary for the initiation of endocardial EMT, it subsequently terminates this process. This dual activity seems to be strictly dose dependent and dynamically controlled in a narrowly defined temporal window. Loss of a single allele of VEGF, or the VEGF164 isoform, results in early embryonic lethality due to underdeveloped endocardial cushions, chamber malformations and impaired vascular development(267;268). Similarly, lowering VEGF levels at E9.5 by hyperglycemia or with a soluble Flt1 chimeric protein inhibits cushion formation and EMT (269). These studies suggest that VEGF expression is required for the endocardial cells to undergo EMT. However, when EMT reaches completion by E10.5, high levels of VEGF expression can be observed in the myocardium of the AV canal (266;270;271). Several lines of evidence suggest that these high levels of VEGF prematurely terminate EMT in endocardial cushions. Of note, a 2-3 fold increase in VEGF production results in mid-gestation lethality, due to heart defects including overdeveloped trabeculae, thin compact myocardium, defective septation and abnormalities in coronary vessels and

OFT remodelling (271). Selective myocardial overexpression of VEGF a day earlier (E9.5 versus E10.5) leads to septal and valve defects due to abnormal expansion of the endocardial cushions (266). These studies were further confirmed by the authors by using the *ex vivo* explants assay where addition of VEGF to E9.5 AV explants almost completely inhibited EMT and collagen gel invasion. Together, these studies show that VEGF signaling plays a critical role throughout endocardial cushion formation, in determining where the cushions will form to initiate EMT and in maintaining EMT to provide enough mesenchymal cells for the remodelling of the valves.

NFATc1 (nuclear factor of activated T cells) is expressed in the endocardium of the heart tube but by E11.5, it becomes restricted to the regions of the future heart valves (272;273). NFATc1 belongs to a family of calcium sensitive transcription factors whose transcriptional activity is dependent on dephosphorylation by calcineurin (274). *NFATc1*-null mice die at E14.5 from congestive heart failure due to lack of endocardial cushion growth and remodelling (272;273). However, EMT occurred normally in *NFATc1*-null embryos, suggesting that NFATc1 is not required for EMT. This observation is supported by the fact that NFATc1 became exported to the cytoplasm as the endocardial cells delaminated from the surface during EMT. Further studies, using chick AV cushion explants, revealed that NFATc1 is required for endocardial cell proliferation and *Cathepsin k* gene expression, which localizes to the valve primordial during later remodelling stages (275). Targeted inactivation of NFATc2, NFATc3 or NFATc4, which are expressed in the myocardium, does not impair valve development (276-279). However, *NFATc2;c3;c4* triple mutants have significant reduction in the number of mesenchymal cells in the AV canal and OFT at E10.5 and have a strong upregulation of VEGF expression (280). The working model suggests that at E9.0, calcineurin/NFAT signalling in the AV canal myocardium inhibits VEGF production, thereby allowing initiation of EMT in response to TGFβ inductive signals. At E10.5, the presence of high levels of VEGF in the AV myocardium leads to EMT termination, endocardial cell proliferation and NFATc1 activation in the endocardium, which will direct valve elongation and refinement.

1.1.7.4.4. ErbB signalling and cardiac jelly

The neuregulins (NGR) are a group of secreted glycoproteins that belong to the EGF family and signal through ErbB receptors, which are a family of tyrosine kinase

transmembrane receptors of the EGFR family. The EGF ligands bind and activate the ErbB receptors, resulting in the formation of homodimers and heterodimers, subsequent autophosphorylation of the cytoplasmic domain and activation of downstream signalling pathways (206). Several lines of evidence suggest that the ErbB signalling is required for heart development including endocardial cushion development. NRG1 expression is restricted to the endocardium, erbB1 has a global expression pattern in embryonic valve tissue, erbB2 is expressed in embryonic hearts, erbB3 is restricted to endocardial and mesenchymal cells of the AV canal and erbB4 is located to the myocardium (145;147;208;281;282). These distinct patterns of expression during cardiac morphogenesis suggest that these molecules may have different functions. Gene targeting studies have revealed that mice lacking *ErbB3* have severe defects in endocardial cushion formation resulting in blood reflux, likely contributing to the lethality observed at E13.5 (281). In contrast, *erbB4*-null embryos die embryonically due to lack of trabeculation and do not have valve defects, suggesting that this molecule is not essential for valvulogenesis (147). *ErbB2* and *NRG1* null embryos die at E10.5 due to lack of trabeculation and they also exhibit underdeveloped cushion and valve tissue, although not as severe as *erbB3*-null embryos (281). These studies suggest that erbB2/erbB3 receptor heterodimers function in the early stages of valve development.

Insight into the mechanism of erbB signalling in cushion development came from studies of the role of hyaluronan synthase 2 (Has2) in AV canal formation. *Has2*-null mice die at E9.5 due to defects in vessel growth, pericardial edema and complete lack of cardiac jelly (192). The authors showed that NRG was able to rescue the phenotype in AV ex vivo explants assays (282). *Has2* null embryos also showed reduced phosphorylation of erbB2 and erbB3 in the endocardial cushions, which was restored upon addition of HA to *Has2*-null explants tissues. These studies suggest that HA in the cardiac jelly regulates erbB2/erbB3 signalling.

1.1.8. Cardiac septation

As discussed in the previous section (1.1.7), endocardial cushion formation is essential for valve development and it also plays major roles in septation of the four chambered heart. The AV canal endocardial cushion is required for the septation of the atria in order to prevent flow of blood from one atrium to the other and failure to do so results in atrial septal defects or patent foramen ovale. Similarly, the inferior AV

cushion is important for the septation of the ventricles and ventricular septal defects arise when this process is disturbed, resulting in mixing of blood between the left and right ventricular chambers. Finally, the OFT cushions are required to divide the systemic and pulmonary blood streams. Thus, defective OFT septation will result in conotruncal defects.

1.1.8.1. Atrial septation

The formation of the atrial septum occurs in the mouse between E10 and E13 (283-285). The first sign of atrial septation is the growth of the primary atrial septum (PAS) from the roof of the atrial compartment, which originates from myocardium near the venous pole (Figure 1.4). As the PAS grows from the atrial roof, mesenchyme from the rim of the pulmonary pit and from the endocardium following EMT will coat the leading edge of the septum and is called the mesenchymal cap (283;286). At this stage, communication between the left and right atria is still visible and is called the ostium primum or atrial foramen. This small communication allows blood to flow through from the right atrium to the left atrium. With further growth of the PAS towards the superior endocardial cushion of the AV canal, the atrial foramen gets smaller and then closes as the mesenchymal cap fuses with cushion tissue of the AV canal after E11.5. Concomitant with this, the AV endocardial cushions start to fuse, thus creating separate left and right AC connections (285;287). Soon after closure, the mesenchymal cap becomes myocardialized by ingrowth of myocardial cells (283).

Before the primary atrial foramen closes, the upper margin of the PAS breaks down or perforates, through programmed cell death, to maintain communication between the atria, which results in the formation of the secondary atrial foramen, or the ostium secundum (Figure 1.4) (283;285;288). The secondary atrial septum, or septum secundum, then develops as an infolding of the dorsal atrial wall on the right side of the PAS. However, it never closes and the opening left below its outer margin is known as the foramen ovale. The remnant of the PAS remains as a flap valve leaflet over the foramen ovale. Prior to birth, well oxygenated venous blood crosses from the right atrium into the left atrium via the foramen ovale for delivery to the systemic circulation. After birth, lung pressure drops and the pressure in the left atrium exceeds that of the right atrium. This change in pressure leads to apposition of this valve leaflet and the secondary septum, followed by fusion and complete sealing of the foramen ovale (283).

Failure to complete this final step results in the formation of a patent foramen ovale (PFO).

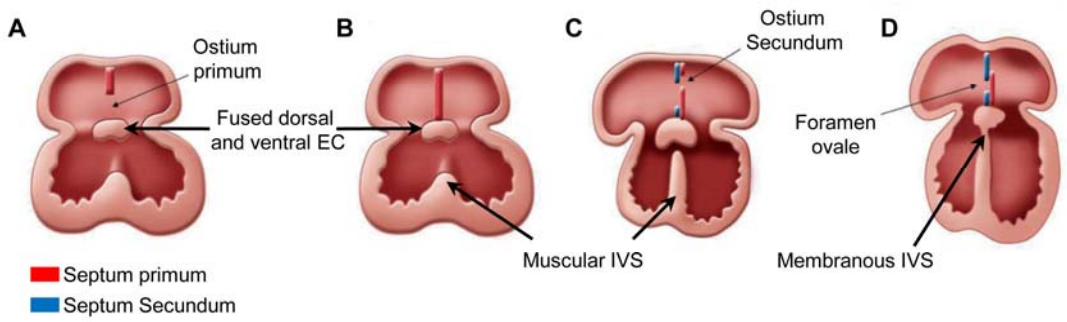


Figure 1. 4. Atrial and ventricular septation

Atrial septation starts with the migration of the septum primum (red) and formation of the ostium primum (A), followed by apoptosis of the septum primum to created the ostium secundum (C) and migration of the septum secundum (blue) (C, D). In the final step, both septum fuse, leaving a foramen ovale (D). At around the same time, ventricular septation begins with the growth of the muscular IVS towards the ECs (A-C). Fusion of the IVS with the ECs closes the interventricular foramen and leads to the formation of the membranous IVS. EC: endocardial cushion; IVS: interventricular septum.

Nkx2.5 was one of the first genes identified in patients with non-syndromic human ASD type II. Interestingly, the role of Nkx2.5 in atrial septal development was highlighted in animal models by the study of Biben and colleagues, where the morphogenesis of the atrial septum in mice heterozygous for *Nkx2.5*-null alleles was analyzed (289). In contrast to humans, it was observed that only a small subset of *Nkx2.5*$^{+/-}$ mice displayed ASD type II. However, other anomalies of the atrial septum such as PFO and atrial septal aneurysm were observed with high frequencies in these mice. Mutations in *Gata4* and *Tbx5* have also been associated with ASDs in human and mouse models and both genes are expressed in the atria during atrial septation (35;290). Of note, *Tbx5*$^{del/+}$ mutant embryos displayed large atrial septal defects, which arises from an absence or reduction of the anterior portion of the septum (119). Moreover, recent studies from our lab demonstrated the importance of the endocardial pathway involving Tbx5, Gata4 and NOS3 in proper atrial septation (291). Interestingly, mice lacking *Tbx5* in endocardial cells developed ASD type II while *Tbx5*$^{+/-}$ mice displayed PFO. It was previously demonstrated that GATA4 functionally interacts with Tbx5 and that disruption of this interaction leads to AVSDs (292). Moreover, a mutation

in *Gata4* that abolishes *in vitro* interactions with Tbx5 has been associated with human ASD. To further test the hypothesis that GATA4 and Tbx5 genetically interact for proper development of the atrial septum, the authors generated compound *Gata4/Tbx5* embryos where *Tbx5* was only deleted in the endocardium (eTbx5). As expected, haploinsufficiency of *Gata4* and *eTbx5* resulted in the presence of large ASDs.

1.1.8.2. Ventricular septation

Following cardiac looping and ventricular chamber expansion, there is a requirement for septation of the chambers to ensure unidirectional blood flow and to maintain different systemic (left) and pulmonary (right) circulations. Septation of the left and right ventricular chambers occurs between E10.5 and E14.5 in mice. The first evidence of septation of the left and right ventricles is the formation of the primary muscular interventricular septum, concomitant with differentiation of the cardiac chambers (Figure 1.4) (284;285). Further septation of the ventricular chambers is dependent on the formation of endocardial cushions in the AV canal and OFT (293).

Different hypothesis have been postulated for the cellular origin of the IVS and a certain degree of uncertainty still remains. Retrospective clonal analyses suggest that the IVS has a dual origin with left and right contributions rather that arising from the left ventricular myocardium only (294;295). Genetic fate mapping indicates that the IVS is derived from cells of the anterior heart field (45). More recent studies have shown that the muscular portion of the IVS originates from the trabecular myocardium (296). These authors showed that the trabeculations of the right and left ventricular walls appose, adhere and fuse just above the interventricular groove. This region becomes more compact as the superior boundary of all trabeculations has fused and results in the initial appearance of the primitive IVS. The IVS has two principal components: an inlet portion that is located near the AV septum and valves and an outlet portion that is derived from the structures nearest the ventricular outflow. Further development and growth of the IVS depends on the expansion of the ventricular cavities as well as trabecular branching (297). As the IVS grows, an apical opening persists between the two ventricles, known as the interventricular foramen. This opening allows mixing of oxygenated and poorly-oxygenated blood in the embryo and must be shut down for proper cardiac function. Closure of the interventricular foramen is brought by fusion of the proximal part of the OFT and the AV endocardial cushions. The last portion of the

septum to be formed is the membranous IVS, which forms from cushion tissue that fills the opening between the AV septum and the muscular septum.

Insight of some of the factors that contribute to the IVS has arisen from studies of human patients that have VSDs. Of note, Tbx5 has been implicated in the formation of the IVS in humans via studies on HOS patients, with the majority having VSDs and ASDs. It has been proposed that the boundary of Tbx5 expression may contribute to the correct positioning of the IVS as evidenced by misexpression of Tbx5 in the ventricular region, which leads in a lack of septum formation resulting in a single ventricle (121). In these embryos, the expression of Hand1/2 was dramatically changed. Interestingly, it was then demonstrated that in *Hand1* knock-in mice, the outer curvature of the right and left ventricle was expanded and formation of the ventricular septum failed (298). This suggest that Hand1 expression at the boundary region between the right and left ventricles may be required for the formation of the interventricular groove and septum. More recently, it was shown that patterning of the IVS is regulated by Tbx5 and Sall4 (299). Sall4 is a member of the Spalt-family of transcription factors and mutations in human Sall4 lead to Okihiro syndrome, which is characterized by VSDs (300). In this study, Sall4 was shown to counteract the activation of the Nppa promoter by Tbx5. Due to its predominant expression in the IVS and pronounced repressor effect in the IVS compared to the working myocardium, this suggest that a boundary of gene expression between the IVS and left ventricle is established. As Tbx5 is able to positively regulate *Sall4* expression in the heart, this in turn leads to increased expression of a co-repressor at the boundary region. In addition, many of the genes involved in ventricular compaction also affect muscular IVS development. This is the case for the *RXRα* knockout embryos, which exhibit a failure to undergo compaction and have a poorly formed ventricular septum leading to defects in ventricular septation (301). Ventricular septation defects also arise in the *erythropoietin* knockout, which is also involved in ventricular compaction (302). Similarly, mice lacking *endothelin-1* have a high prevalence of VSDs (303). In conclusion, ventricular septal defects are seen frequently in complete and/or conditional knockout models, possibly due to the action of transcription factors in chamber formation or endocardial cushion formation as well as combinatorial interactions between these genes in these processes.

1.1.8.3. Outflow tract septation

Initially, the outflow tract (OFT) connects to the embryonic right ventricle with the aortic sac around E8 in the mouse. Concomitant with the formation of the ventricular chambers during looping morphogenesis (E9-9.5), the OFT rapidly increases in length by addition of myocardial cells originating from the secondary heart field (38-40;42).

Several signalling pathways, including Wnt, Shh, TGFβ and FGF have been shown to be important regulators of SHF cell behaviour. Their roles in SHF proliferation, differentiation and recruitment to the heart tube are reviewed in section 1.1.1. In addition, several studies have revealed the importance of a transcriptional network in SHF development. A possible start point for this network is Islet1 (*Isl1*), which is present in the anterior lateral and pharyngeal mesoderm (44). Lineage tracing studies have shown that *Isl1* descendants contribute the right ventricle and OFT as well as the atria and the left ventricle. *Isl1*$^{-/-}$ embryos lack the OFT and right ventricle and the atrial posterior region is reduced, thus suggesting an important role of Isl1 in OFT development (44). Moreover, mutant embryos displayed reduced expression of BMP and FGF, which could possibly explain the reduced proliferation observed as these growth factors are involved in cell proliferation and survival of cardiac progenitors. A number of studies have established that other transcription factors, including Mef2c, Tbx20, Tbx1 and Hand2, are required in the transcriptional program controlling SHF development. For instance, *Mef2c*$^{-/-}$ embryos have impaired OFT and right ventricle development, suggesting that Mef2C is required in SHF development (304). Furthermore, it has been shown that *Mef2c* is regulated by multiple enhancers that govern a subset of its endogenous expression. One of these enhancers directs expression exclusively to the SHF and derivatives in the OFT and right ventricle (305). Interestingly, the function and activity of this enhancer is dependent on conserved Isl1 and Gata4 binding sites, suggesting that Isl1 and GATA factors may cooperate to activate a transcriptional program in the SHF. In addition, FoxH1 and Nkx2.5 can also regulate expression of the *Mef2c* gene in the SHF, although on a different enhancer (306). This same study also demonstrated that *FoxH1*-mutant embryos form a primitive heart tube but fail to form the OFT and right ventricle, indicating that FoxH1 is essential for OFT development. Thus, it appears that these pairs, Isl1/Gata4 and Foxh1/Nkx2.5, may be at the top of the hierarchical order controlling SHF development.

In addition to Mef2c, Isl1 is also able to activate an Nkx2.5 enhancer, together with Tbx20 and Gata4 (307). A role for Tbx20 in OFT development has also been inferred based on the observation that partial knockdown of *Tbx20*, using RNA interference, results in impaired OFT and right ventricle development, leading to DORV, PTA and right ventricle hypoplasia (307). Moreover, mutations in *Tbx20* results in a hypoplastic, unlooped heart that has an hourglass appearance (140;142;307). The interpretation of this phenotype varies but it seems that Tbx20 does not primarily function in the SHF; rather, it is thought that the observed hypoplasia is due to collapse of SHF function. Tbx1, another member of the T-box family, also plays critical roles in OFT development in humans and mice. Indeed, *Tbx1* mesodermal ablation recapitulates the OFT abnormalities characteristic of the *Tbx1* haploinsufficiency phenotype, suggesting that mesodermal Tbx1 is necessary and sufficient to support normal septation, growth and alignment of the OFT (308). Recently, Srivastava and colleagues identified and enhancer from the *Tbx1* gene that is sufficient to direct expression to the SHF; this enhancer is activated by Fox2A, FoxC1 and Fox2C through consensus Forkhead-binding sites in the enhancer (309). Moreover, Tbx1 is implicated in the regulation of Fgf8 and Fgf10, which play important roles in proliferation of the SHF (61;310). Of note, *Fgf8*$^{-/-}$ embryos lack and OFT and right ventricle and have reduced expression of Isl1 in the pharyngeal mesoderm, suggesting that FGF8 plays a key role in the induction of cardiac progenitors in the SHF (61). Thus, FGF8 is implicated in the hierarchy for SHF development, possibly upstream of Isl1. Taken together, these observations suggest that a very specific genetic network regulates OFT development.

During lengthening of the OFT, two different regions can be discerned, a proximal (conus) and a distal portion, which are separated by a distinct bend (311;312). Endocardial cushion formation is essential, not only for aortic and pulmonary valve development, but also for the septation of the OFT into a systemic and pulmonary circulation, which ensure adequate oxygenated blood circulation from the aorta to the rest of the body and return of non-oxygenated blood to the lungs through the pulmonary artery. The endocardial cushions are bulges of cardiac jelly populated by a mixture of mesenchymal cells that migrate into the OFT from the pharyngeal area and by mesenchymal cells generated by EMT of endocardial cells. Furthermore, these endocardial cushions, which can be distinguished into a septal and parietal cushion, are

continuous throughout the OFT, spiralling around one another. At around the same time, neural-crest derived mesenchymal cells descend from the 3rd, 4th and 6th branchial arches to the distal endocardial cushions and collect as condensed rods of mesenchyme in the distal OFT cushions but do not extend into the conus although a cluster of neural crest cells populate the conal cushions (Figure 1.5) (313;314). These prongs of condensed mesenchyme are connected to the aortic sac by the 4th and 6th arch arteries. Together with tissue in the aortic sac, they form the aorticopulmonary septation complex, which has been suggested to play key roles during initial separation of the single outflow vessel to form the ascending aorta and pulmonary trunk (311). The next important step in development is fusion of the septal and parietal cushions, which divides the distal OFT into the intrapericardial components of the aorta and pulmonary trunk (312;315). In this way, the aortic blood flow within the OFT exits via the arteries of the 4th aortic arches while the pulmonary flow exits via the 6th arch arteries.

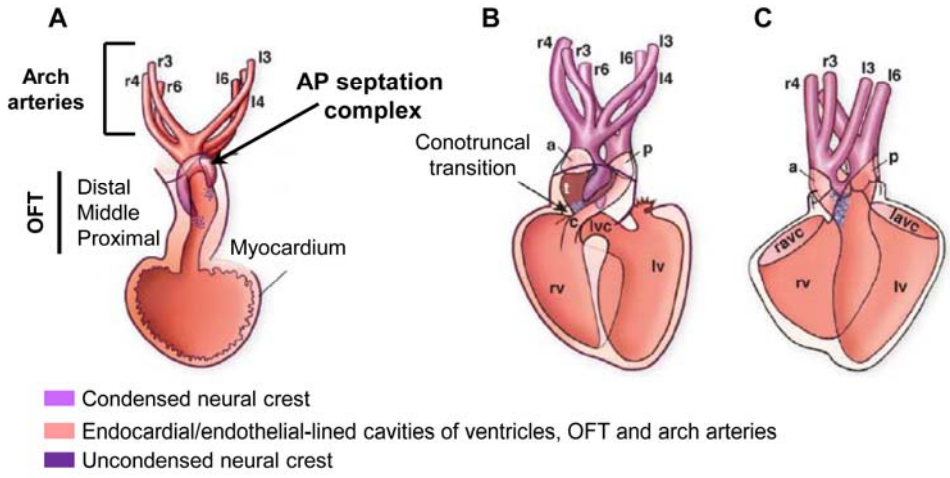


Figure 1. 5. Schematic representation of neural crest during septation of the OFT

(A) Representation of the condensed neural crest mesenchyme (purple) of the aorticopulmonary septation complex. (B) Septation of the distal and middle portions of the OFT by the aorticopulmonary septation complex. (C) Septation of the proximal part of the OFT, which leaves a seam of cardiac neural crest cells. OFT: outflow tract. Adapted from Hutson M and Kirby M, *Heart development and regeneration*, 2010: 441-462.

After the rods of condensed mesenchyme have triggered the onset of fusion in the distal portion of the OFT, the proximal outflow septum closes in a zipper-like fashion

from distal to proximal towards the ventricles. In addition to the cushions that have fused to separate the distal portion of the proximal OFT into prospective aortic and pulmonary components, two further intercalated cushions have grown in the common OFT (284;312). One of the intercalated cushions, positioned anteriorly, forms a leaflet and sinus of the aortic valve whereas the posterior intercalated cushion forms the comparable components of the pulmonary valve. The other two leaflets of the semilunar valves are derived from the cushions that have fused to separate this distal part of the proximal OFT. The proximal cushions within the proximal OFT eventually close concurrently with invasion of the cushions by myocardium (316;317). This process is referred to as myocardilization and is the result of invasion of the endocardial cushion by cardiac myocytes existing within the walls of the proximal OFT; this causes the cushions to bulge and meet into the lumen. When these bulging cushions touch, the endocardium covering the proximal cushions breaks down, allowing mixing of the underlying mesenchyme and myocardium, which brings about fusion of the opposing cushions to form a septum within the ventricular OFT. After this process is complete, the mesenchyme that formed the endocardial cushions is remodelled into the aortic and pulmonary valves.

Cardiac neural crest (CNC) cells are essential for proper septation of the OFT. Much of what we know today about the role of CNC cells in heart development has been learned from the chick ablation model (85;314;315;318). Ablation of the CNC cells leads to a number of cardiovascular phenotypes including defective development of the OFT, abnormal patterning of the great arteries and abnormal myocardial function. The morphological defects associated with defective OFT development are overriding aorta and complete absence of septation leading to persistent truncus arteriosus (PTA). One of the earliest defects observed in the CNC cell ablation is abnormal heart looping. This occurs before arrival of CNC cells into the OFT and thus, may explain conotruncal defects such as DORV and transposition of the great arteries TGA, which result from defective rotation of the OFT. Recent studies have revealed that neural crest cells are able to modulate the SHF. Interestingly, Waldo *et al* demonstrated that cells of the SHF failed to join the proximal OFT following ablation of neural crest cells leading to a shorter OFT, thus confirming the previous hypothesis made by the same group (42;85;319). Thus, CNC cells seem to exert an important influence on septation of the OFT and on its rotation as well, through modulation of the SHF.

Targeted and/or conditional deletion of several genes in mice is able to recapitulate all or portions of the neural crest ablation phenotype in chick. Some of these genes are directly expressed in CNC cells whereas others are expressed in the SHF and can regulate NC cells migration, proliferation or survival (Table 1.2) (320-328). For example, Splotch mutation encoded by the *Pax3* gene, which is highly expressed in the neural tube and migrating NC cells mimics NC ablation. *Splotch* mutants die by E14.5 because of myocardial dysfunction and have conotruncal defects including PTA, DORV and abnormal patterning of aortic arch arteries (329;330). Moreover, inactivation of *GATA6* in vascular smooth muscle (VSM) cells or neural crest cells leads to interrupted aortic arch and PTA, again recapitulating portions of the NC cell deficiency (331). Interestingly, GATA6 was shown to regulate *Sema3C* in the OFT and VSM cells and a similar cardiac phenotype is observed in *Sema3C* null mouse embryos (331;332). On the other hand, neural crest involvement is well established in DiGeorge syndrome characterized by PTA, interrupted aortic arch, absent or hypoplastic thymus and craniofacial dysmorphology (333;334). The gene responsible for this syndrome maps to chromosome 22, in a region that encodes *Tbx1*. However, *Tbx1* is expressed in the pharyngeal endoderm and SHF mesenchyme but not in the CNC cells (335). It has been suggested that Tbx1 may be able to regulate Fgf8 in the SHF, which affects some aspects of neural crest development; and this in turn, may explain the OFT defects observed in *Tbx1* deficient mice.

1.2. Origin and fate of cardiac lineages

The myocardial, endocardial, epicardial and cardiac neural crest cells perform specialized functions that are required for proper functioning and integrity of the heart. Eventhough the hypothesis of a common ancestral origin for these cells is still debated, studies have demonstrated that they originate from the cardiac mesoderm, proepicardial organ and neural crest cells (Figure 1.6). In this section, I shall discuss the origin and function of the cell types that make up a heart.

1.2.1. Myocardial lineage

The cardiac progenitors are among the first lineages to be established in the embryo. Tremendous efforts have been directed towards identifying the origin and location of the cardiac progenitors. Fate mapping as well as lineage tracing studies have revealed that the cardiomyocyte progenitors are located in the precardiac

Gene	*Expression*	*Phenotype*	*Function*	*Reference number*
Pax3	neural crest cells	PTA, outflow misalignment, DORV, VSD, abnormal patterning of aortic arch arteries	Required for migration of CNC cells	329
Tbx1	pharyngeal ectoderm and endoderm	Hypoplasia of the distal OFT, PTA and aortic arch patterning defects	Supports proliferation of cells in the SHF	310, 335, 522
Fgf8	pharyngeal endoderm	PTA, outflow misalignment, DORV, VSD, abnormal patterning of 4th pharyngeal artery	Survival factor for neural crest cells	59, 60
Sonic hedgehog	pharyngeal endoderm	PTA and abnormal arch arteries	Proliferation of SHF	69, 70
Gata6	vascular smooth muscle cells and SHF	PTA and interrupted aortic arch	Regulates morphogenetic patterning of the OFT and aortic arch	331
Sema3C	OFT and subpulmonary myocardium	PTA and interrupted aortic arch	Migration of CNC cells	332
Gata3	OFT	PTA, DORV, VSD and anomalies of the aortic arch	Patterning of the OFT	706
Hand2	CNC cells	misalignement of the OFT and aortic arch artery defects	Regulates proliferation and differentiation of the SHF cells	848
Foxc1/Foxc2	SHF and CNC cells	Lack of OFT	CNC cell survival during their migration to the heart	320
Msx1/Msx2	neural crest cells	DORV, PTA, TOF	Neural crest cell survival	321
Bmpr1a	neural crest cells	PTA and short OFT	Myocardial proliferation	322
Alk2	neural crest cells	PTA and arch artery patterning defects	Neural crest migration	328
Alk5	neural crest cells	Abnormal remodelling of aortic arch arteries and PTA	Important in the post-migratory CNC cells	323
Disheveled 2	CNC cells	DORV, TGA and PTA	Survival of CNC cells	324
RALDH2	SHF	PTA and abnormal arch arteries	Patterning of the CNC cells before migration	50
Cx43	Neural crest	PTA and VSD	Neural crest cell migration	325
Pitx2	SHF and CNC cells	PTA, DOVR and TGA	Regulates arterioventricular alignment	104, 327

Table 1. 2. Genes involved in outflow tract septation

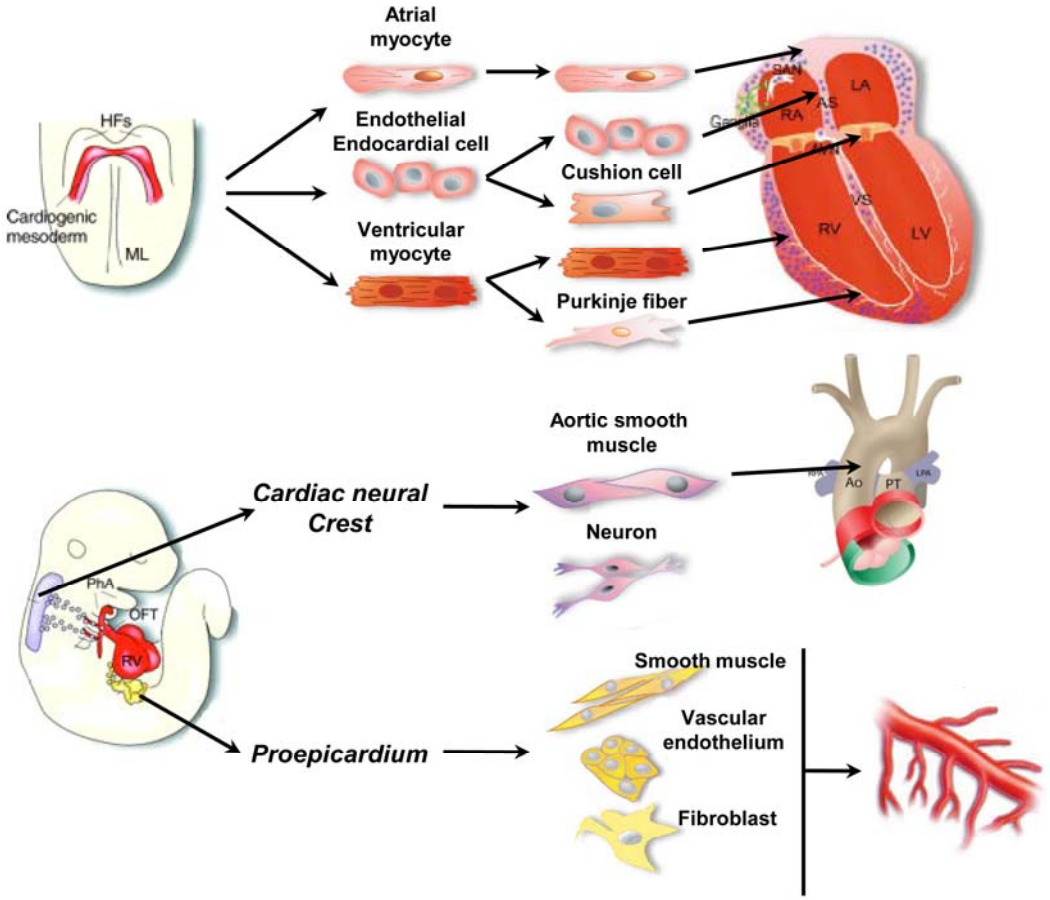


Figure 1. 6. Origin of cardiac lineages

The figure shows that cardiac cell types arise through the linage diversification of the embryonic three precursor pools in the mouse heart: the cardiac mesoderm, neural crest or proepicardium. Ao: aorta; AS: atrial septum; HF: heart fields; LA: left atrium; LV: left ventricle; ML: midline; OFT: outflow tract; PhA: pharyngeal arches; PT: pulmonary trunk RA: right atrium; RV: right ventricle; SAN: sinoatrial node; VS: ventricular septum. Adapted from Laugwitz KL *et al*., Development. 2008, 135: 193-205.

mesoderm (336). In addition, several studies have provided evidence that the endocardial, myocardial and epicardial lineages are derived from a multipotent progenitor cell, which is covered more in details in section 1.2.2.2 and 1.2.3.1 (337-340). The cardiomyocytes are striated muscle cells that are composed of numerous myofilaments that run parallel along the axis of the cells and provide the basis of the cell's ability to contract. In the mouse, cardiac actins and myosins are first visible at E7.5-8.0, right before the first contractions of the heart tube (341;342).

Transplantation experiments in both chick and mouse embryos have revealed that the cardiogenic region contains inductive signals that promote myocardial differentiation (1;343). With the use of mouse models and culture system, including embryonic stem cells, P19 cells and rat cardiomyocytes, several growth factors like BMPs and FGFs, have been found to be required for cardiac differentiation. Thus, different signalling pathways will transmit inductive signals to the cardiac mesoderm, leading to the induction of numerous transcription factors including the transcription factors *Nkx2.5*, *Gata4*, the T-box factors, *SRF*, *Mef2c*, *Hand1* and *Hand2* (12;72). Interestingly, BMPs have been implicated in cardiomyocyte differentiation. This is due, in part, to the observation that in zebrafish, *Bmp2b* mutants have reduced or absent expression of *Nkx2.5*, which is present in the cardiac mesoderm (344). Moreover, in *Xenopus*, blocking Bmp signalling with a dominant negative ALK3, a truncated BMP receptor or an inhibitory smad6, prevents progression to a state of terminal differentiation rather than blocking expression of cardiac markers (17;345). It was also demonstrated that activation of Bmp signalling in mouse ES cells as well as P19 cells is able to promote cardiac differentiation and expression of cardiac transcription factors (346;347). There is also accumulating evidence that FGF signalling plays a role in differentiation of cardiac lineages. For example, mouse ES cells lacking *FGFR1* have downregulation of mesodermal genes, leading to defective cardiomyocyte differentiation (348). It was also shown, in differentiating mouse EC cells, that the concomitant addition of Bmp2 and FGF2 to the culture media enhances the expression of several cardiac marker genes including *Nkx2.5*, *Mef2C*, *Gata4* and *αMHC* (349).

1.2.2. Endocardial lineage

The endocardium is the endothelial, innermost cell layer of the linear heart tube. The endocardium develops from endothelial cells that form bilaterally paired tubes by

the process of vasculogenesis. Early in development, these bilateral tubes fuse in the midline to form a single tubular cardiac compartment, which lies beneath the foregut endoderm and is covered ventrally by splanchnic mesoderm. The endocardium plays essential functions during heart development. As discusses earlier, endocardium-myocardium interactions are important for the formation of trabecular myocardium and for differentiation of myocytes into the Purkinje fibers of the CCS (350;351). Furthermore, endocardial cells lining specific regions will undergo EMT to form endocardial cushions at the AV canal and OFT (216;352). These cushions will then give rise to the atrioventricular and semilunar valves, the membranous portion of the IVS, to the atrial septum and to the division of the OFT into separate aortic and pulmonary trunks (216;353). Despite all of these essential functions, much remains to be learned about the origin and the development of the endocardium.

1.2.2.1. Origin of the endocardium

Whether endocardium and myocardium share a common progenitor in the cardiac mesoderm remains controversial; so far, two models have been proposed. In the first model it is believed that the cardiogenic precursors are prespecified prior to their migration through the primitive streak. Consistent with this, retroviral single cell tagging and tracing experiments have demonstrated that individual labelled cells give rise to a clone consisting of myocardium or endocardium but never both (354-357). Similarly, lineage tracing experiments in zebrafish demonstrated that the individual labelled cells in the heart field never contribute to both myocardium and endocardium (358). Moreover, myocardial precursors are spread throughout the heart field whereas the endocardial progenitors are restricted to the ventral marginal region. These studies suggest that the separation of these two lineages occurs at the blastula stage, prior to formation of the mesoderm.

In contrast, there is growing evidence that endocardium and myocardium are derived from a common multipotent mesodermal progenitor. Consistent with this, fate-mapping studies in mice indicate that cells expressing *Mesp1* and *Flk1* contribute to both endocardium and myocardium (359;360). Furthermore, Cre-mediated lineage tracing of *Isl1*+ and *Nkx2.5*+ cardiac populations, which are first apparent in the cardiac crescent, suggest that they contribute to both myocardial and endocardial cells as well as aortic endothelium (44;361). Similarly, when the *Cre* is expressed under the control

of the SHF-specific regulatory elements from the *Mef2c* gene, both endocardial and myocardial lineages are labelled (45). Using an *NFATc1-nuc-lacZ* reporter mouse, Misfeldt *et al* showed that the endocardium is specified as a cardiac cell lineage independent from other vascular populations. They also established that endocardial cells are derived from an *Flk1+* multipotent cardiovascular progenitor, providing further evidence that endocardium and myocardium are derived from a common precursor (362). Interestingly, it was shown that the QCE-6 cell line, which is derived from gastrulating explants of quail embryos, can differentiate into both endocardial and myocardial cells, suggesting that a common endocardial/myocardial precursor also exists in chicks (363).

1.2.2.2. Endocardial differentiation

The initial step in endocardial formation is the delamination of the endothelial precursor cells from the precardiac mesoderm. Initiation of endocardial precursor cell formation from the mesoderm was shown to be mediated by TGFβ, but not VEFG (364). TGFβ1/2/3 were able to stimulate endocardial cell formation as evidenced by the increased invasiveness of mesenchymal cells from mesodermal explants. However, VEGF did not induce mesenchymal cell formation but rather stimulated a monolayer of endothelial cells to grow out from the precardiac mesoderm (364).

Zebrafish GATA5 plays a major role in endocardial differentiation as evidenced in *faust* mutants, which maps to the *Gata5* locus producing a truncated protein that acts as a dominant negative transcriptional regulator (33;365). In the mouse, *Gata5* is largely restricted to endocardial cells, which suggests that it may be required for specific aspects of endocardial development (366). Further studies from our lab indicate that Gata5 is induced concomitant with endocardial differentiation and that blocking *Gata5* with antisense RNA blocked the ability to form endocardial cells (367). These studies were done using a mesodermal cell line derived from the hearts of polyomavirus large T-antigen transgenic mice that can differentiate into endothelial cells upon retinoic acid treatment (368). Using this model system, our group was able to show that differentiation of these cells leads to downregulation of early myocardial markers including Gata4, Twist and Tbx20 (367). Concomitant with this, the endocardial phenotype appeared and expression of Gata5, Flt1 and NFATc1 was induced. Furthermore, it was demonstrated that Gata5 is induced prior to other differentiation

markers including Tie2, ErbB3 and connexin 37. NFATc1 is essential for endocardial development and is expressed specifically in the endocardium, starting at the time of initiation of differentiation at E7.5 (272). The Tie2 receptor is also important for endocardial development as evidenced by the endocardial defects in *Tie2*$^{-/-}$ embryos (369). As the endocardial cells become terminally differentiated, other markers including endothelin-1, tenascin X, epicardin and Epas1 are upregulated (367). However, our understanding of endocardial development from the precardiac mesoderm still remains obscure and further studies will be required to shed light on this process.

1.2.3. Epicardial lineage

Prior to E9.5, the heart is composed of two layers, an outer myocardial and an inner endocardial layer. At E10.5, the third layer, the epicardium, migrates towards the developing heart and envelops the myocardium. The epicardium consists of a single layer of flat mesothelium connected to the myocardium by subepicardial connective tissue. Lineage tracing studies in avians have demonstrated that the epicardium originates from an extracardiac mesodermal cell population, called the proepicardium (370). The proepicardium protrudes from the pericardial mesothelium covering the sinus venosus in the direction of the tubular, looped heart. In avians, the proepicardial organ develops multiple finger-like protrusions, or vili, into the pericardial coelomic cavity whereas studies in fish and mouse suggest that the proepicardium generates short protrusions or blebs that transform into proepicardium cysts (371;372).

1.2.3.1. Common progenitor with myocardium

The epicardium contributes the majority of nonmyocardial cells in the heart. In the last decade, it has been reported that proepicardial cells are derived from *Nkx2.5*-expressing progenitors and contribute to the formation of cardiomyocytes (373-375). Consistent with this, explanted proepicardial cells can spontaneously differentiate into beating cardiomyocytes in the presence of BMPs and FGFs (376;377). These observations suggested that the porepicardium is derived from the cardiac mesoderm rather than the septum transversum. More recently, it was demonstrated that the SHF can contribute to both the myocardium and the proepicardium (378;379). In this study, a small group of cells expressing *Tbx18* in the splanchnic mesoderm were labelled 1 day before induction of proepicardium formation. Twenty four hours later, expression

was observed both in the inflow tract and in the proepicardial organ. FGF signalling via Mek1/2 was required to separate the epicardial lineage from the precardiac mesoderm whereas Bmp signalling was important for myocardial differentiation.

1.2.3.2. Development of epicardial cells

The proepicardial organ become morphogenetically identifiable by E8.5. Between E9.5 and E10.5, the majority of proepicardium vesicular cells aggregate and are released into the epicardial cavity (380;381). These vesicles then migrate and attach to the myocardial surface, where they contribute to the formation of the primitive epicardium. During proepicardium extension, a subpopulation of proepicardium and epicardial cells undergo an epithelial-to-mesenchymal transformation and subsequently migrate into the subepicardial space (381). Epicardial EMT, which occurs between E11.5 and E12.5 in the mouse, is observed at the AV junction, in the ventricular epicardium, and at the junction between the ventricles and OFT but not in the atrial epicardium (382-384). These transformed cells, termed epicardium-derived cells (EPDCs), then migrate into the myocardium and differentiate into a variety of myocardial cell types, including subepicardial mesenchyme, interstitial fibroblasts, coronary endothelium, coronary smooth muscles and hemangioblast. In chick embryos, EPDCs will invade the myocardium in a spatio-temporal fashion, starting at the inner curvature at HH19, followed by the AV canal, atria, ventricles HH23/24 and finally will invade the OFT at HH30 (381;385). Once within the myocardium, the EPDCs will migrate to their destination.

Factors that have been shown to be involved in regulation of epicardial EMT are transcription factors and growth factors Slug and Snail, Ets-1 and Ets-2, the Wilms tumor gene WT-1, the adhesion molecules E-cadherin, α4 integrin, FGF, TGFβ, PDGF and VEGF (386-395). For example, inhibition of Ets-1 and Ets-2 by using antisense oligonucleotides was shown to result in loss of subepicardial mesenchyme formation and to prevent formation of coronary vessels (391). In addition, Fgf2 and VEGF but not PDGF-AA, PDGF-AB or Fgf1 were shown to induce EMT of a rat proepicardial cell line (396). These observations are consistent with the idea that epicardial EMT is regulated by paracrine signals emanating from the myocardium.

1.2.3.3. Epicardium and myocardial patterning

The epicardium secretes mitogenic factors that are required for normal compact zone myocardial growth and architecture (397). In avians embryos, in which ablation or delayed formation of the epicardium was observed, the ventricles had persistent thin walls (389;398;399). Moreover, removal of the epicardium in chick hearts resulted in decreased cardiomyocyte proliferation (400). A number of mouse gene mutations have been shown to result in poorly-formed and thin walled ventricles, which eventually leading to embryonic lethality by E14 (Table 1.3) (401-405). This highly suggests that formation of the compact zone is critical for embryo survival.

It should be noted that the thin walled ventricle phenotype does not necessarily imply that the gene mutated in that study functions in the epicardium or myocardium. However, there are some clear examples where genes play a primary role in epicardium-myocardium interactions. Interestingly, WT1 is expressed in the epicardium, in the subepicardial mesenchyme and in migratory epicardium-derived cells but is lost in differentiated EDPCs (389;390). In mouse embryos lacking *WT1*, the proepicardium does not form properly, resulting in lack of subepicardial mesenchymal cells and formation of a thin wall ventricle. Embryos die from heart failure possibly due to accumulation of blood in the pericardial cavity (406). Several WT1 target genes have been identified, including E-cadherin, α-integrin 4, erythropoietin and the neutrophin receptor TrkB (407-410). These genes have all been associated with epicardium formation, epicardial cell differentiation or epicardium-myocardium interactions, which establishes WT1 as an important regulator of epicardium formation.

In addition, it has been demonstrated that RA signalling is required within the epicardium for proliferation of the myocardium. Ablation of the RA receptor gene *RXRα* or epicardial-specific deletion of *RXRα* leads to severe hypoplasia of the compact zone (301;411). Similarly, loss of *Raldh2* in mice leads to profound myocardial hypoplasia (412). RA signalling has been shown to induce expression of the FGFs in the epicardium (400;413). *Fgf9* is expressed in the epicardium and endocardium at E10.5 in mice but by E12.5, its expression becomes restricted to the endocardium (414). Similar expression patterns are seen with *Fgf16* and *Fgf20* (414). The FGF signal is received by the FGF receptors (FGFR) 1 and 2, which are expressed in the myocardium. FGF signalling, through FGFR1 and FGFR2, was shown to induce

Gene	*Cardiac phenotype*	*Reference number*
Gata4	VSD, DORV, myocardial thinning	122, 123, 731
Fog2	Thin venticular myocardium, common AV valve and TOF	488
WT1	Thin ventricle; trnasmural bleeding into the pericardial cavity	406
Fgf9	Small hearts with thin myocardium	414
Fgf16	Chamber dilatation, atrial and ventricular wall thinning, poor trabeculation	401
β-catenin	Cardiomyocyte hypoproliferation and thin myocardium	402
Erythropoietin	Ventricular hypoplasia, epicardium detachment and abnormal vascular network	302
Erythropoietin receptor	Ventricular hypoplasia, epicardium detachment and abnormal vascular network	302
Raldh2	Reduced ventricular compact layer and altered coronary vessel development	403
β-4 intergin	Cardiac failure, epicardium and coronary vessels fail to form	404
VCAM-1	Cardiac failure, epicardium and coronary vessels fail to form	404
Hexim-1	Thin ventricular walls and abormal coronary vessel patterning	405
RXRα	Thin ventricular wall, lack of ventricular compaction,	411

Table 1. 3. Phenotypes of genes associated with thin ventricular walls

cardiomyocyte proliferation (399;414). Furthermore, in the absence of RA and FGF signalling, the myocardial cells undergo premature differentiation and fail to proliferate (414). Thus, these observations indicate that reciprocal FGF signalling between the epicardium-myocardium is important for formation of the compact zone.

1.2.3.4. Development of the vasculature

The formation of functional coronary vessels over the heart is essential for normal cardiac development. Development of the coronary vasculature has been well-described in avians (415-417). These studies demonstrated that coronary vessel development occurs in a wave-like pattern, originating from the atrial-ventricular groove and going towards the ventricular apex. Coronary vessel development starts as a subset of epicardial cells undergo EMT at stage 26 in chick and E11.5 in mouse (384). This process leads to the formation of mesenchymal cells in the subepicardial space between the epicardium and myocardium. These endothelial-derived cells self-assemble to form vascular channels that are later ensheathed by smooth muscle cells and perivascular fibroblasts. The vascular plexus then grows by angiogenesis to provide circulation on a one-to-one basis by covering the heart. Gene targeting studies have demonstrated that the FGF and Hedgehog signalling pathways are required for the formation of the coronary vasculature, mainly by regulating expression of VEGF and angiopoietins (418). Lavine *et al* showed that FGF signaling triggers a wave of hedgehog activation that progresses from the atrial-ventricular groove (E12.5) to the apex of the ventricles (E13.5) (418). Hedgehog signals from the epicardium, in turn, signal to cardiomyocytes and perivascular mesenchymal cells to induce expression of *Vegf-A*, *Vegf-B*, *Vegf-C* and *Ang2*, resulting in the formation of the coronary plexus. The last step in coronary vessel development is ingrowth of small vascular channels into the base of the aorta, which coalesce to form a single coronary stems in the left and right coronary sinuses (419;420).

1.2.4. Cardiac neural crest linage

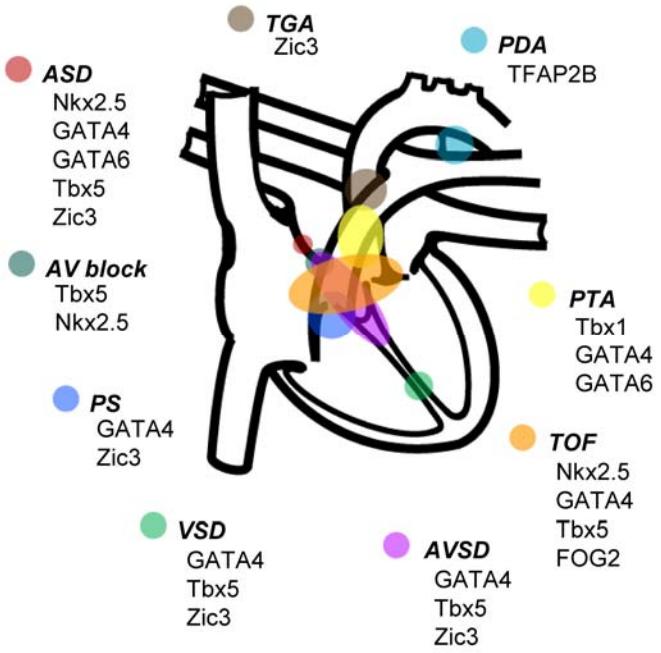
The neural crest cells are multipotent cells that originate from the neuroepithelium (384). Crest cells are divided into cranial and trunk, based on axial origin. They migrate widely throughout the embryo during development. A sub-region of the cranial neural crest has been called the cardiac neural crest (CNC) due to their importance during heart development (314;421;422). These cells originate from the neural plate

between the axial boundaries of the otic placode and somite 3 and migrate into the 3rd, 4th and 6th branchial arteries. As mentioned earlier, CNC cells invade the heart through the arterial and venous poles, where they give rise to the parasympathetic innervations to the heart, the smooth muscle layer of the great arteries, the endocardial cushions of the OFT and the aorticopulmonary septum that divides the OFT into a systemic and pulmonary circulation (see section 1.1.8.3) (423;424).

In addition to contributing to the septation of the OFT, CNC cells are required for patterning and remodelling of the great vessels. This is evidenced by the lack of variable combination of the arch arteries derived from the branchial arches in consequence to the ablation of NCC in chick embryos (318). Neural crest-specific deletion of *PDGFRα* leads to normal induction and migration of CNC cells, however, the 3rd, 4th and 6th are arteries were dilated or reduced in diameter compared to controls (425). Conditional ablation of both *PDGFRα/β* in the neural crest gave similar results with complete penetrance in addition to reduced migration of the NC cells into the OFT (426), suggesting cooperative interactions between these two isoforms in recruitment of NC cells to the OFT.

1.3. Congenital Heart disease

Congenital heart disease (CHD) refers to any malformation of the cardiovascular system that is present near or at the time of birth (Figure 1.7) (427-438). In human, CHD is the most common developmental defect, representing an estimated 25% of all congenital malformations. CHD is estimated to occur at a prevalence of 4-50 per 1000 live births and is believed to be the leading cause of death in the first year of life (439). Several epidemiologic studies have established that CHD is a heritable trait. Even though tremendous advances have been made in the diagnosis and treatment of CHD over the years, it still carried significant mortality and morbidity. Isolated cases of CHD are not uncommon but most CHDs are associated with genetic syndromes such as DiGeorge, Williams-Beuren, Alagille, Noonan and Holt-Oram syndromes (439). CHD has an increased risk of recurrence within families; among affected relatives, the cardiac phenotypes are usually different and occur with variable penetrance and expressivity, likely indicating that modifying factors including genetic and environmental, influence the phenotype (440).



Gene	Human phenotype	Reference
Nkx2.5	ASD, AV block, VSD, TOF, HCM	168, 427, 469, 517
Tbx1	DiGeorge syndrome	526, 527
Tbx5	HOS, ASD, AVSD, AV block, TOF	464, 465
Tbx20	ASD, TOF Valve defects	474, 555, 556
Gata4	ASD, VSD, AVSD, TOF, PS	127, 470, 754
Gata6	ASD, TOF	473, 779,780
FOG2	TOF, DORV	428, 429
JAG1	Alagille syndrome, TOF, PS	430, 431, 811
Notch1	BAV, calcification	453, 454
MYH6	ASD	432
PTPN11	Noonan syndrome, PS, ASD, AVSD, HCM	433 434, 435
Zic3	Heterotaxy, ASD, AVSD, TGA, PS, DORV	436
TFAP2B	Char syndrome, PDA	437, 438

Figure 1. 7. Cardiac anomalies associated with transcription factor mutations in human

ASD: atrial septal defect; AV: atrioventricular; AVSD: atrioventricular septal defect; PDA: patent ductus arteriosus; PS: pulmonary stenosis; PTA: persistent truncus arteriosus; TGA: transposition of the great arteries; TOF: tetralogy of Fallot.

Despite the significant efforts of the past decade to elucidate the genetic basis of heart defects, the causative genes for the majority of CHD remain unidentified. Several modifying factors, including both genetic, environmental factors and teratogens, have been shown to influence the phenotype (440). Why is there so little information regarding the genetic basis? Cardiac development is a complex integration of various pathways, structures, cell types, gene networks and regulatory components. However, in the past decade, tremendous progress has been made in understanding and elucidating the molecular events that govern cardiac morphogenesis. With the help of several model system such as frogs, zebrafish, mice and chick, it has been possible to identify a plethora of genes required for normal heart development and dissect the hierarchy of these genetic programs.

The majority of cardiac malformations affect the valves and septa, which originate from endocardial cells. These include valvar abnormalities, abnormal communications between the chambers (atrial septal and ventricular septal defects) and endocardial cushion defects. Other types of CHDs include conduction defects, hypoplastic ventricles and defects of the myocardium.

1.3.1. Bicuspid aortic valve

Bicuspid aortic valve (BAV) is the most common CHD, affecting 1-2% of the population, with a 2:1 ratio of males to females (441;442). It may be silent during life, however, at least one third of individuals with BAV will develop serious complications, including valvular stenosis, regurgitation, infective endocarditis and dilation, dissection or rupture of the aorta (442;443). It is estimated that 54% of all valve replacement surgeries on people over the age of 50 years can be attributed to BAV disease (444). Eventhough these statistics are astounding, BAV may still be grossly underestimated as it may remain asymptomatic in childhood and even into adulthood and thus, no imaging studies are ordered. Furthermore, populations studies have suggested that BAV may be responsible for more mortality and morbidity than any other CHD combined (445). BAVs may appear as an isolated defect or it can be associated with other cardiac congenital malformations, in particular coarctation of the aorta, interruption of the aortic arch and ventricular septal defects (446). Genetic studies have established that BAV is a heritable trait with autosomal dominance and incomplete penetrance (447). However, the mechanisms underlying the formation of BAV remain poorly understood.

The leaflets of the aortic valve develop from the endocardial cushion tissue of the OFT. In a normal individual, the aortic valve possesses three leaflets whereas patients with BAVs have only two leaflets (Figure 1.8). In human, the anatomy of the BAV usually includes leaflets (cusps) of unequal size due to the fusion of two cusps leading to one larger cusp, the presence of a central raphe (usually present in the middle of the greater cusp) and smooth cusp margins (441). The raphe actually represents the location where the two cusps fused during valvuloseptal development. Furthermore, leaflet orientation varies widely among patients, with fusion of the right and left (R-L) coronary leaflet being more common, occurring in 59% of cases whereas union of the right and noncoronary (R-N) leaflet occurs in 37% of BAV cases (448). Interestingly, a more recent study carried by Fernandes S *et al* showed that the R-N BAV is associated with a greater risk and more rapid progression of having moderate to severe aortic stenosis and aortic regurgitation and a shorter time to valve intervention (449).

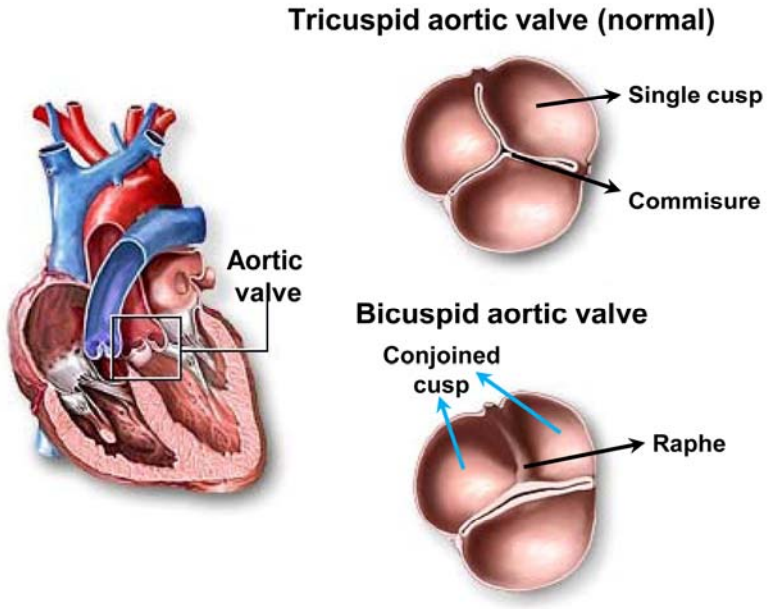


Figure 1. 8. Bicuspid aortic valve

Schematic representation of a normal aortic valve and a bicuspid aortic valve (BAV). The aortic valve allows oxygen-rich blood to flow from the left ventricle of the heart to the rest of the body. The BAV develops during the early weeks of pregnancy from abnormal valvular development.

In animal models, the morphology of the BAV has been unraveled using Syrian hamster, which have a natural incidence of BAV when inbred. It was discovered that

fusion of the left and right valve cushions at the beginning of valvulogenesis is the key factor in the formation of BAV, suggesting that the morphology is predetermined prior to the end of valve development (450). To gain more insight into the etiology of BAV, Fernandez B *et al* studied the *eNOS* null mice and Syrian hamsters (451). In the *eNOS*$^{-/-}$ mice, the BAV occurred from the fusion of the right and noncoronary leaflet (R-N) and is thought to be the result of defective development of the OFT endocardial cushions prior to OFT septation. In Syrian hamsters, the fusions occurred from the right and left coronary leaflet (R-L), which is thought to results from anomalous septation of the proximal portion of the OFT, possibly caused by wrong behavior of neural crest cells. Thus, at least in animal models, the R-L and R-N BAVs seems to have different etiological entities.

Based on numerous studies performed on BAV patients and their first-degree relative, it has been demonstrated that this disease follows an autosomal dominant inheritance with reduced penetrance (452). Although some cases of BAV are sporadic, familial clusters have been identified with an incidence of 10-17% in first-degree relative, which suggests that a genetic component is involved. This is further supported by comparing the incidence of BAV in first-degree relatives with the general population (1-2%). As the aortic valve originates from the endocardial cushions of the OFT, it is strongly accepted that a mutation in a gene involved in one or more aspects of endocardial cushion formation may be responsible for this disease. So far, only mutations in the *Notch1* gene in human have been found patients with BAV (453-455). In these patients, nonsense mutations as well as frameshift mutations were identified, suggesting haploinsufficiency. In addition, mutations in *KCNJ2* (potassium inwardly-rectifying channel, subfamily J, member 2) which belongs to a large family of protein that produce potassium channels, have been linked to the Andersen syndrome and were also found in association with BAV and BAV with coarctation of the aorta (456). In search for potential candidate genes that could lead to BAVs, Mohamed S et al reviewed the morphogenesis of the semilunar valves and came across the *UFD1L* gene, which encodes a component of a multienzyme complex involved in the degradation of ubiquitin fusion proteins (457). UFD1L is highly expressed in the cardiac OFT during embryogenesis and is located on chromosome 22q11.2, which is commonly associated with CHDs that involve the development of neural crest cells. Consistent with this, attenuation of *UFD1L* transcripts in chick CNC cells resulted in OFT septation defects,

suggesting that it plays a role in CNCs cells during conotruncal septation (458). Mohamed S *et al* demonstrated, by doing fluorescent in situ hybridization, that the *UFD1L* gene product was significantly reduced in BAVs that were obtained from patient surgery. Thus, this supports the hypothesis that BAV is a genetic disorder, with *UFD1L* being a good potential candidate gene. Therefore, it is possible that impaired behavior of CNC cells may lead to reduced degradation activities, which subsequently results in impaired aortic valve formation causing fusion of the valve cushions.

Animal studies have also been very helpful to find potential candidate genes that could underlie BAV formation. Of note, a null mutation in *NOS3* and *Gata5* in mice was revealed to result in the formation of BAVs, with a penetrance of 32% and 25% respectively (451;459;460). Moreover both of these studies demonstrated the formation of the BAV occurred from fusion of the right and noncoronary leaflet, leading to a R-N BAV. Interestingly, this suggests that the R-N BAV is the result of a morphogenetic defect that arise before OFT septation, potentially involving a defect in endocardial cell differentiation, defective EMT or reduced migration of mesenchymal cells into the endocardial cushions. The mechanism that underlie the formation of BAV is still unknown. These two mouse models represent an important step in understanding the potential mechanism that leads to BAV formation during valvuloseptal development and also represent a powerful tool to investigate the mechanism.

In conclusion, BAV is heritable and is associated with serious complications later in life. Although valve surgery can replace the BAV, relatively little is known regarding the mechanism that leads to BAV formation. Valve development is a complex process and remodeling of the ECM during patterning of the aortic valve is a critical part of the development of the OFT. Clarification of the genetic cascade underscoring normal valve development may provide crucial information regarding the pathogenesis of BAV. Moreover, very few genes have been associated with BAV in humans. The studies provided here show that we are moving in the appropriate direction but there is still much to be done.

1.3.2. Communication between the chambers

Abnormal communication between the chambers may result from defective atrial ventricular or atrioventricular septation. In the case of atrial septal (ASD) and ventricular septal (VSD) defects, there is shunting of blood from one chamber to the other, with direction of flow being determined by the pressure gradient (Figure 1.9). A more complex cardiac malformation may also arise from defective septation of all three regions, creating a common mixing chamber and will be discussed in section 1.3.3.

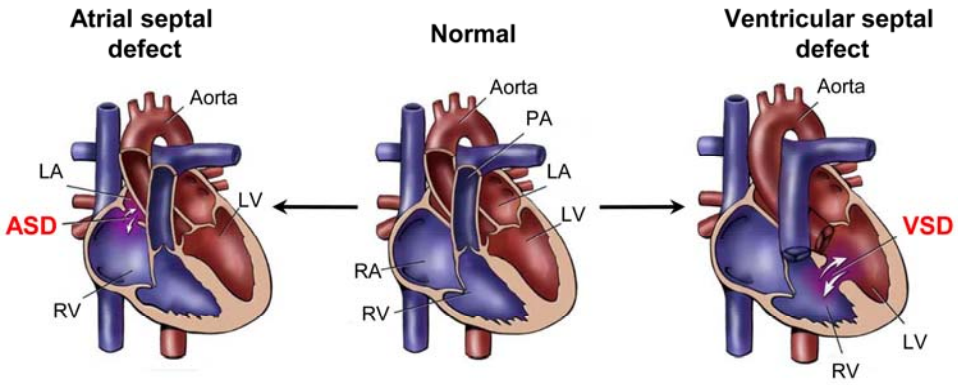


Figure 1. 9. Atrial and Ventricular septal defects

Defective septation of the atria leaves a hole, known as atrial septal defect while defective septation of the ventricles leads to ventricular septal defects, allowing blood to flow from one cavity to the other. ASD: atrial septal defect; LA: left atrium; LV: left ventricle; PA: pulmonary artery; RA: right atrium; RV: right ventricle; VSD: ventricular septal defect.

1.3.2.1. Atrial septal defects

Atrial septal defects (ASDs) account for about 10% of all CHDs and are characterized according to their location: an ostium secundum type, which results from non-closure of the foramen ovale; an ostium primum defect, an ostium secundum defect and a sinus venosus defect (461). The primum ASD occurs when the septum primum does not fuse with the AV endocardial cushion leaving the ostium primum open. This defect may be associated with defective formation of the mesenchymal cap or with the AV cushions. If the AV cushions are defective, the primum type will be considered as an endocardial cushion defect, which is discussed in section 1.3.3. Secundum ASD is the most common type of ASD in children and results from defective closure of the ostium secundum. This occurs when the septum secundum fails to grow properly or when the ostium secundum is too large, which prevent the septum secundum from

completely covering the opening. In addition, ASD type II is often associated with right atrium and right ventricle dilatation. The sinus venosus defect results from failure of the sinus venosus to be incorporated into the right atrium.

ASDs can be found in isolation and also in some patients with certain syndromes, including Holt-Oram, Ellis-van Creveld and Noonan syndromes (462). Mutations in several genes involved in cardiac development have been associated with familial and sporadic types of ASDs (463). Mutations in several transcription factors required for normal heart development have been linked to human ASDs. Mutations in *Tbx5* have been associated with Holt-Oram syndrome, an autosomal dominant disorder characterized by skeletal abnormalities affecting the thumbs or entire arm (464-466). *Tbx5* mutations have also been identified in humans with non-HOS malformed hearts (467). Similarly, mutations in *Nkx2.5* have been identified in patients with sporadic ASD or ASD associated with progressive AV blocks or complex CHDs such as tetralogy of Fallot (168;169;468;469). Furthermore, *Gata4* mutations have been found in both familial and sporadic cases of ASDs (127;470-472). More recently, mutations in *Gata6* have been found in patients with ASDs as well as tetralogy of Fallot (473). Finally, mutations in Tbx20 were identified in patients with ostium secundum ASD (474).

1.3.2.2. Ventricular septal defects

VSDs are basically openings or holes in the ventricular septum (475). VSDs can be seen in isolation (20%) but are frequently present in children with Holt-Oram syndrome as well as other syndromes, including DiGeorge, Alagille and Noonan syndromes, accounting for 50% of cases (439;476). VSDs can be classified according to their location, including the following: perimembranous, muscular, inlet and subpulmonary subtypes. The most common form of VSD is the perimembranous type, which involves the membranous septum and some of the adjacent muscular septum. This subtype is located just beneath the septal leaflet of the tricuspid valve and is subtended by the aortic valve. When the VSD is small, there is little or no hemodynamic disturbance of the left ventricle, which results in a small left-to-right shunt with no pulmonary hypertension. However, when the VSD is large, an equal pressure is observed in the two ventricles and the direction of the shunt will be dictated by the relative resistance of the systemic and pulmonary circulations.

Several human syndromes or sporadic cases of VSDs have been found with mutations in the same genes as they are associated with ASDs, notably *Nkx2.5*, *Tbx5* and *Gata4* (169;469;477-479). In animal models, mutations in a large number of genes involved in heart development have been associated with VSDs in animal models, usually in association with other cardiac abnormalities. These include conditional and/or complete knockout models of *Nkx2.5*, *Tbx5*, *Gata4*, *Hey2* and *FOG2* (247;315;480-483).

1.3.2.3. Endocardial cushion defects

Atrioventricular canal defect, also known as atrioventricular septal defect, covers a spectrum of congenital heart malformations characterized by failure of proper development of the endocardial cushions in the embryonic heart (484). Such malformations involve one or all of the following: atrial, ventricular and/or atrioventricular septal defect as well as the adjacent leaflets of the two AV valves. In complete atrioventricular canal (CAVC) defect, all four chambers communicate due to the presence of an ostium ASD, a VSD and a common AV valve (Figure 1.10) (287). This results in interatrial and interventricular systemic-to-pulmonary shunt, thus increasing the ventricular pressure, volume overload and pulmonary hypertension.

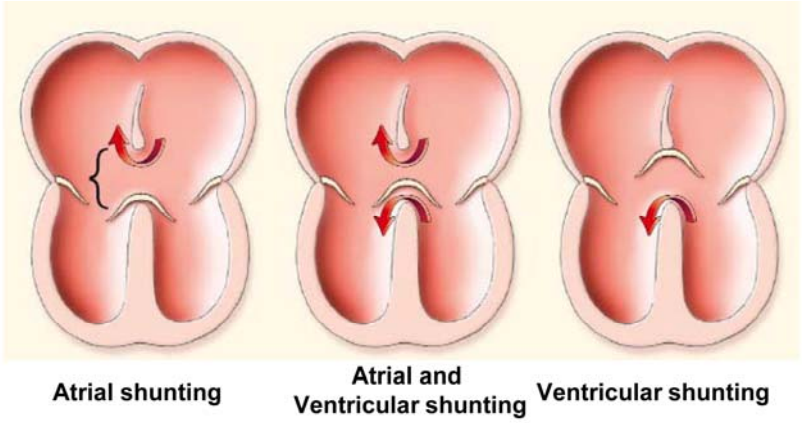


Figure 1. 10. Different types of shunting associated with AVSD

The potential for shunting in AVSD (bracket) depends on the relationship between the bridging leaflets and the ventricular and atrial septum. Adapted from Anderson R *et al*., *Cardiac development and regeneration*, 2010: 255-277.

The common AV valve is basically built up of four to five leaflets (superior, inferior, mural in the right and left ventricle and antero-superior), embryonically derived from the endocardial cushions. CAVC occurs in 2 out of every 10,000 live births and account for 3% of all cardiac malformations. CAVC defect is commonly associated with other cardiac anomalies and 80% of children born with CAVC will die within the first two years of life due to congestive heart failure.

CAVC defects are most commonly seen in patients with Down syndrome (Trisomy 21) (485). The genetic basis of CAVC still remains elusive but genes involved in endocardial cushion formation are likely candidates. The *Down syndrome critical region 1* (*DSCR1*) gene is present on the human chromosome 21 and the syntenic region of mouse chromosome 16 and has been linked to the Trisomy 21 phenotype. During valvuloseptal development in the heart, DSCR1 is expressed in the developing AV and semilunar valves as well as the IVS and ventricular myocardium (275). Moreover, its expression co-localizes with NFATc1 in the developing valve endocardium and NFAT-rich calcineurin response elements have been found adjacent to exon 4 of the *DSCR1* gene. Expression of DSCR1 is absent in *NFATc1*$^{-/-}$ embryos and its expression in Ts16 mice co-localizes with abnormal valvuloseptal development. This suggests that NFATc1 signalling is required for DSCR1 expression in the developing endocardium. Other mouse models such as the *Cx40*$^{-/-}$*Cx43*$^{+/-}$ and *Cx40*$^{-/-}$ *Cx45*$^{+/-}$ mice, have been observed with common AV valves (486;487). These connexins are expressed in the conduction myocardium and since this region is derived from the AV canal, it is possible that development of this specialized myocardium is impaired. The presence of common AV valves is also observed in *Fog2*$^{-/-}$ and *Gata4*$^{ki/ki}$ mice, which are both required for expression of myocardial genes (488;489). Similar defects were observed in *Gata4/Tbx5* and *Gata4/Gata5* compound mutants, indicating that genetic interactions between transcription factors is important for cardiac development (292;490).

1.3.3. Conduction defects

The cardiac conduction system relays the electrical impulses through the heart to achieve a coordinated and fast contraction. As detailed in section 1.1.6, a complex genetic network is required for the development of the CCS. Thus, loss of transcription

factor regulation during cardiac development can have detrimental consequences on the contraction of the heart, leading to conduction defects.

1.3.3.1. Atrioventricular conduction disease

The AV conduction system is comprised of specialized cells that allow synchronized cardiac excitation, which results in contraction of the atria and ventricles. In the adult heart, the AV conduction system is made up of the AV node, the His bundle, the right and left bundle branches and the Purkinje fibers. AV conduction disease, or AV block, therefore occurs when conduction is slowed or blocked along one of the components of the AV conduction system such that the electrical continuity between the atria and ventricles is impaired (491). Classification of the AV conduction disease depends on the extent of the block (first, second or third degree) as well as the site of the block (above or below the His bundle). Moreover, the extent is based on characteristics of the PR interval, which is measured from the onset of P-wave to the onset of QRS complex. First-degree AV block is a mild form of conduction delay, resulting from prolongation of the PR interval. In presence of a second-degree AV block, some atrial impulses are conducted to the ventricle while other are not. Second AV block can be further subdivided into type 1 or type 2. In type 1 block, the PR interval becomes progressively prolonged before the appearance of the AV block while in type 2 AV block, the block occurs without any prolongation of the PR interval (491). In the most severe form, referred as third-degree AV block, no atrial impulses conduct to the heart and thus, the QRS complex occurs independent of the P-wave on an electrocardiogram.

Familial clustering of AV blocks has established that it shows an autosomal dominant mode of inheritance (492). Moreover, AV conduction disease has been associated with CHDs. Of note, mutations in human Nkx2.5 gene were identified in patients with AV conduction disease as well as other forms of CHDs, including ASDs, VSDs, tetralogy of Fallot and tricuspid valve abnormalities (168;169). In humans, Nkx2.5 mutations cause AV node conduction delays that progressively worsen during postnatal life such that advanced second-degree and third-degree blocks are observed in the third decade of life (169). In addition, ventricular restricted inactivation of Nkx2.5 in mice leads to progressive AV blocks due to a hypoplastic AV node (171). A similar phenotype was also observed when Nkx2.5 was deleted beginning at two weeks of life,

suggesting that Nkx2.5 is necessary for proper cardiac conduction and contraction (493).

1.3.3.2. Long QT syndrome

Long QT syndrome is a genetic conduction disease characterized by a prolonged QT interval on the electrocardiogram, syncope, T-wave anomalies, ventricular tachycardia and torsades de pointes (irregular heart beat), all of which can lead to sudden death (494). As for many CHDs, Long QT syndrome occurs with variable expressivity and incomplete penetrance and can be subdivided into two primary clinical categories: acquired and inherited. The acquired form generally results from pharmacological therapeutic intervention, often to treat disorders unrelated to cardiac dysfunction (495). Moreover, acquired forms may also result from other rhythm disorders, cardiac ischemia and some cardiomyopathies. The inherited form of long QT syndrome is predominantly autosomal dominant, although some recessive forms exist but are associated with more severe phenotypes.

Changes in the QT interval is caused by an altered action potential, which consists of a depolarisation, plateau and repolarisation phases that reflect the electrical activity across the cardiomyocyte during one contraction. To date, about 12 genes have been associated with the inherited, or congenital, form of the long QT syndrome. These include the sodium, calcium and potassium channels as well as accessory subunits and associated modulatory proteins, which are all required for the generation of the action potential (494;495). Sodium channel dysfunction in long QT syndrome are mostly due to mutations in the *SCN5A* gene. *SCN5A* encodes the protein Nav1.5, which conducts the sodium inward current and is responsible for the initial depolarisation of cardiomyocytes (496). About 65% of mutations identified in SCN5A are located in exons 20-28 and produce a persistent sodium current, which delays repolarisation (497). Following identification of LQT causing mutation in SCN5A, several other genetic disorders were associated with SCN5A mutations, such as the sick sinus node syndrome, conduction disease, Brugada syndrome, atrial fibrillation and cardiomyopathies (496).

1.3.4. Disease of the myocardium

Hypertrophic cardiomyopathy (HCM) is a common inherited cardiac disease of the myocardium characterized by a marked thickening of the left ventricular wall. The

prevalence of HCM is 1/500 individuals and is the most prevalent genetic cardiovascular disease transmitted as an autosomal dominant trait (498;499). In most cases, the diagnosis relies on electrocardiographic and echocardiographic demonstration of left ventricular hypertrophy. HCM is a major cause of premature sudden cardiac death among the young individuals and healthy athletes. Furthermore, some individuals with HCM remain asymptomatic throughout life while others exhibit progressive exercise intolerance and heart failure or sudden cardiac death. It may also be associated with left ventricular outflow tract obstruction, diastolic dysfunction, myocardial ischemia and atrial fibrillation (498).

Anatomically, HCM can manifest with negligible to extreme ventricular hypertrophy, minimal to extensive fibrosis and myocyte disarray. HCM is the first myocardial disease in which the genetic basis was identified. To date, more than 450 different mutations have been identified, most of which are missense mutations, within 24 genes encoding sarcomeric, calcium-handling and mitochondrial proteins (499;500). Most mutations were observed to occur in two genes, *Myh7* that encodes β-myosin heavy chain and *Mybpc3* which encode myosin binding protein C. Over the years, researchers have begun to identify pathways that link sarcomere pathology to myocyte growth and cardiac hypertrophy. The functional consequences of gene mutations have been well studied both *in vitro* and *in vivo*. For example, the Arg403Glu mutation in βMHC was shown to disrupt sarcomere assembly when transfected into adult feline cardiomyocytes (501). Moreover, functional studies using muscle biopsy specimens from patients carrying this mutation have demonstrated that the muscle fibers have depressed velocity of shortening, reduced force to stiffness ratio and reduced power output (502). In conclusion, dissecting out the components of intracellular signalling cascades triggered by a gene mutation that subsequently results in cardiac hypertrophy is of great interest and may lead to the identification of new targets for therapeutic intervention.

1.4. Transcription factors involved in heart development

During the past decade, an emerging body of evidence has accumulated that transcription factors control specific aspects of cardiac development and play critical roles in transcriptional regulation during cardiogenesis and during the adaptive process

in the human heart. This section will describe some of the most important players in cardiac morphogenesis.

1.4.1. NK-2 class homeodomain proteins

A novel paradigm in cardiac development originated from the discovery of the *tinman* gene in Drosophila, which is the founding member of the *NK-2* class of homeobox genes. Flies lacking the *tin* gene lack all dorsal mesodermal derivatives, including the dorsal vessel, visceral muscle and a subset of body wall muscles (503;504). The most striking phenotype of tin mutants was the complete lack of cardiac tissue. Since the discovery of *tinman* in Drosophila, many vertebrate homologues have been discovered. To date, ten *tinman* homologues have been found in diverse vertebrate species: *Nkx2.1* to *Nkx2.10*. The defining feature of the NK-2 family is the presence of a homeodomain, which consists of a 60 amino acid DNA binding motif. In addition, Nk-2 family members can be grouped into three classes based on the presence of two conserved domains, the tin/Nkx2.5-domain (TN domain) that mediates protein interactions and the NK-2 specific domain (NK2-SD) that functions as a repressor (505).

The developmental role of Nkx2.5 in the heart has been extensively studied in *Xenopus*, zebrafish and mice. In *Xenopus* or zebrafish embryos, overexpression of *Nkx2.5* leads to the formation of hyperplastic hearts, characterized by thickening of the myocardium due to an increase in the number of myocytes (506;506). Furthermore, transplanted cells expressing higher levels of *Nkx2.5* started to express cardiac markers, although these cells did not beat, suggesting and important role for this gene in cardiac cell fate decision and for proper patterning within the heart field. Targeted deletion of the *Nkx2.5* gene in mice leads to embryonic lethality by E9-10 from cardiac insufficiency (118;507). The heart tube formed normally but failed to undergo correct looping, a single ventricular chamber was present and the OFT was truncated. In contrast to *tinman*-mutant flies, Nkx2.5 is not essential for initial cardiomyocyte specification as beating cardiomyocytes were present in the linear heart tube. Possibilities for functional redundancy could account for this less severe phenotype, but this is unlikely as no other mammalian *NK-2* gene has been identified with an expression pattern overlapping that of Nkx2.5 in early heart development. Consistent with this, murine *Nkx2.6* is transiently expressed in the posterior myocardium, sinus

venosa and dorsal pericardium at E8.5 and in the OFT myocardium at E9.5 but no cardiac abnormalities were observed in *Nkx2.6*-null mice (508;509).

Recent lines of evidence suggest that Nkx2.5 is also important in perinatal hearts, as demonstrated by the rapid conduction and contraction defects observed within 4 days using tamoxifen-inducible *Nkx2.5* knockout mice, leading to premature death (510). Deletion of *Nkx2.5* starting at two weeks of age resulted in first degree AV block and heart enlargement similar to perinataly loss of Nkx2.5, suggesting that Nkx2.5 is important for proper conduction and contraction (493). In addition, mice lacking ventricular *Nkx2.5* develop progressive and advanced conduction defects as well as left-ventricular hypertrophy postnatally, with 50% of mutant animals progressing to complete heart block by 12 months (171). This is similar to what is observed in human where heterozygous mutations of *Nkx2.5* cause various cardiac anomalies and progressive conduction defects as well as occasional left ventricular dysfunction (168;169;511;512).

In search for direct downstream target genes of Nkx2.5, the promoter activities of potential candidate genes were explored. Among those examined, Nkx.25 was found to regulate the *Nppa* promoter as well as other genes including *cardiac α-actin*, *Mef2c*, *connexin 40*, *myocardin*, *sodium-calcium exchanger 1*, *endothelin-converting enzyme-1* and *CARP* (513). These genes encode structural proteins and transcriptional regulators that play important roles in cardiomyocytes. Connexin 40, for example, is a major constituent of the gap junctions and loss of *Cx40* leads to conduction defects (514;515). Interestingly, transgenic mice overexpressing human *Nkx2.5* harbouring the Ile183Pro mutation (in the homeodomain) develop AV conduction defects, which are accompanied by reduced expression of the gap junction proteins Cx40 and Cx43 (516). In addition, Nkx2.5 is able to transactivate the Cx40 promoter, together with Tbx5 (119). These observations likely suggest that Nkx2.5 plays an important role in the cardiac conduction system, in part, through the regulation of gap junction proteins. However, further investigations are required to elucidate the molecular and cellular mechanism of how Nkx2.5 regulates the development of the cardiac conduction system as well as other aspects of cardiac development.

To date, a total of 38 heterozygous *Nkx2.5* mutations have been reported, which are associated with a wide range of CHDs including VSD, TOF, DORV, tricuspid valve

anomalies and progressive conduction defects (168;469;511;517;518). The importance of *Nkx2.5* in atrial septation and the conduction system is illustrated by the observation that ASDs are associated with 68.4% (26/38) mutations while 65.7% (25/38) of mutations identified result in AV block. Further studies have also indicated that 4% of CHDs are due to heterozygous *Nkx2.5* mutations. Among the heterozygous mutations found in humans, 12 are within the homeodomain and are believed to impair the transcriptional activities of Nkx2.5 due to reduced DNA-binding affinities. Haploinsufficiency is postulated to be the underlying mechanism of disease.

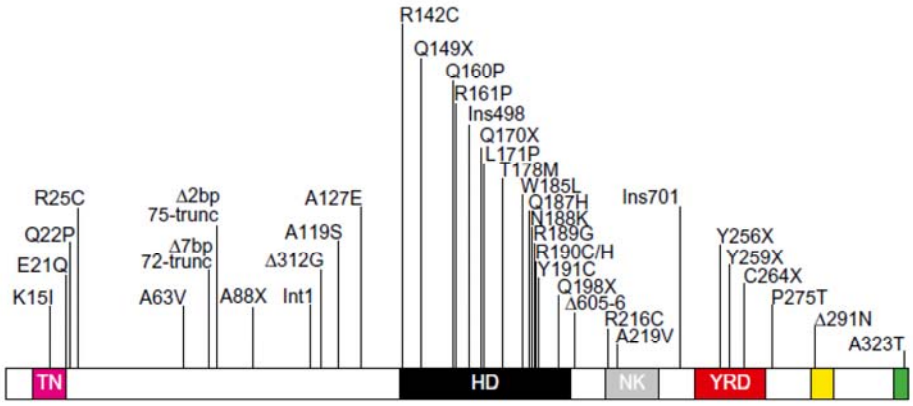


Figure 1. 11. Localization of human Nkx2.5 mutations associated with CHD

Mutations are represented on the Nkx2.5 protein, showing the conserved and functional domains. HD: homeodomain; NK: NK2 specific domain; TN: tinman/Nkx2.5 domain; YRD: tyrosine rich domain. From Elliot D *et al*., *Cardiac development and regeneration*, 2010: 569-597.

1.4.2. T-box transcription factors

The vertebrate T-box (Tbx) gene family includes around 20 different members. Tbx proteins are characterized by the presence of a highly conserved 180 amino acid DNA binding domain termed the T-box. The T-box domain is relatively large, generally comprising about a third of the entire protein, and individual members of the family show varying degrees of homology across this region. *Brachyury* (*T*) is the founding member of the T-box gene family and was identified in 1990 based on a short tail phenotype in mice (519). Since then, additional members of the T-box gene family have been identified in vertebrate and invertebrate organisms from hydra to humans. Among the different members of the family, Tbx1, Tbx2, Tbx3, Tbx5, Tbx18 and Tbx20

have been shown to be expressed in the cardiovascular system, where they play important roles in formation of the OFT, valve and conduction system development, chamber formation and maturation and epicardial development (Figure 1.12A). The specific roles of these T-box factors is discussed in the following section.

1.4.2.1. Tbx1

The Tbx1 gene was originally cloned from a E12.5 mouse cDNA library using the DNA-binding domain of *brachyury* as probe. Evidence for a role for Tbx1 in heart development came from the finding that the region of the human chromosome 22q11.2, where *Tbx1* maps, is often deleted in patients with DiGeorge syndrome. DiGeorge syndrome is characterized by a variety of abnormalities, including absence or hypoplasia of the thymus, cleft palate, facial dysmorphism and cardiac anomalies mostly of the outflow tract, including interruption of the aortic arch, VSD, pulmonary atresia and persistent truncus arteriosus (520;521). Targeted disruption of *Tbx1* in mice phenocopies important aspects of DiGeorge syndrome, including OFT defects (522-524). Therefore, it was suggested that Tbx1 plays an important role in OFT morphogenesis. *Tbx1* expression can be detected in the pharyngeal endoderm, the mesodermal core of the pharyngeal arches and the SHF. Consistent with a role in SHF, mesodermal-specific deletion of *Tbx1* recapitulates the OFT defects seen in *Tbx1*-null embryos (335;525). The phenotype also includes reduced cell proliferation, which may underlie the reduced contributions of the SHF to the OFT of *Tbx1*-deficient mice. Time course deletion experiments further demonstrated that Tbx1 is required for OFT development between E8.5-9 and E9.5, coinciding with the contribution of SHF cells to the OFT (335).

In recent years, mutations in human *Tbx1* have been associated with DiGeorge syndrome. Almost all of the mutations identified in *Tbx1* map to the C-terminal transactivation-repression domain and an additional mutation results in deletion of the nuclear localization signal (526;527). To date, the implications of these mutations on heart or cranofacial development has not been reported. In conclusion, Tbx1 is the only gene for which mutations have been found in some patients without the chromosomal deletion. As many birth defects, including many CHDs, are derived from problems of the pharyngeal system, Tbx1 is an excellent tool to probe the genetic network that govern embryonic pharyngeal development.

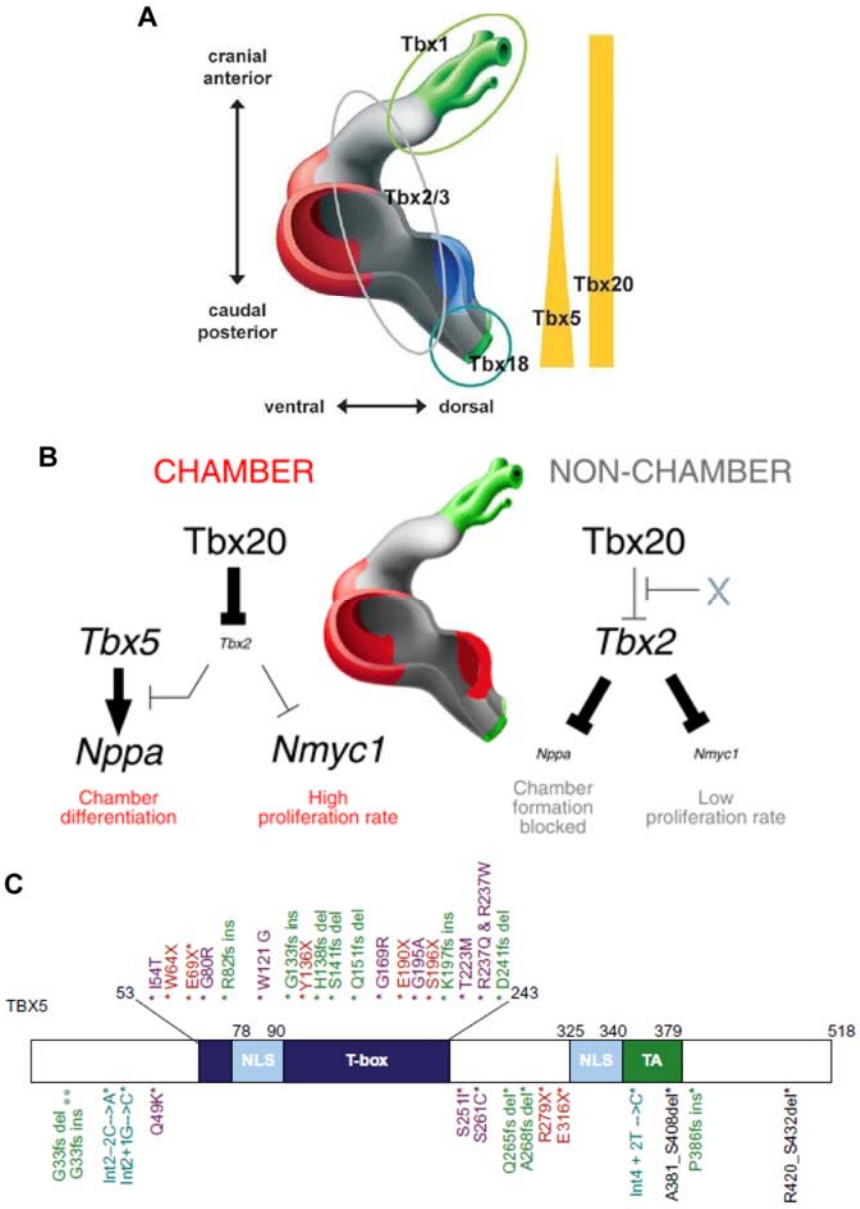


Figure 1. 12. Role of T-box factors in heart development

A) Schematic representation of an E9.5 heart showing expression of the different T-box factors in the heart. Tbx1 is expressed in OFT, Tbx18 in the sinus horns and Tbx2/Tbx3 exert their function in the non-chamber myocardium. The yellow bars represent the expression of Tbx5 and Tbx20. Modified from Hoogaars WMH *et al*., *Cell. Mol. Life*, 2007. 64:646-660. B) Model of T-box factor regulation of cardiac chamber development. From Stennard F and Harvey R, *Development*, 2005. 132: 4897-4910. C) Mutations in human Tbx5 associated with Holt-Oram syndrome. NLS: nuclear localization sequence; TA: transactivation domain. From Conlon F and Yutzey K, *Cardiac development and regeneration*, 2010: 651-671.

1.4.2.2. Tbx2 and Tbx3

In the same homology-based screen that identified Tbx1, two additional T-box members were identified, namely Tbx2 and Tbx3. In the mouse, *Tbx3* is expressed in the inner cell mass and extraembryonic tissues early in development, and in the limbs, mammary buds, spleen, pituitary gland, lung, kidney and heart (528). Shortly after the identification of *Tbx3*, it was reported that mutations in this gene are associated with the ulnar mammary syndrome (UMS), a human disorder characterized by malformation of the upper limb and hypoplasia of apocrine and/or mammary glands (529;530). In the heart, *Tbx2* is broadly expressed, including in the myocardium of the AV canal, endocardial cushions, SHF and neural crest cells (125;131;528).

Within the T-box family, *Tbx2* and *Tbx3* share extensive regions of homology across the entire protein and function as transcriptional repressors . Indeed, they were shown to act as powerful repressors of the *Nppa* and *Cx40* promoters *in vitro*, which are normally activated by *Tbx5* and *Nkx2.5* (Figure 1.12B) (131;166). Furthermore, these studies demonstrated that Tbx2 competes with Tbx5 for the TBE element on the *Nppa* promoter in non-chamber myocardium, forming a repressive complex with Nkx2.5. Several studies have shown that Tbx2 is able to repress the expression of chamber specific genes including *ANF*, *Cx40* and *Cx43* in tissue culture and transgenic mouse models, suggesting that Tbx2 functions in cardiac development to repress the gene programs associated with chamber formation and differentiation (125;531). Consistent with this, null deletion of *Tbx2* results in abnormal development of the AV canal and defects in OFT alignment (132). Furthermore, ectopic expression of ANF was found in the AVC of *Tbx2*-null embryos.

Tbx2 expression overlaps with that of *Tbx3* in the heart primordia, posterior primitive heart tube, AV canal and conduction system, with a stronger and broader expression for Tbx2. This suggests possible functional redundancy as well as distinct functions for Tbx2 and Tbx3. Consistent with this, Tbx2 and Tbx3 are able to repress the same target genes, including *Nppa* and *Cx40*. *Tbx3*-null embryos die at midgestation over a range of several days due to yolk sac anomalies in addition to hindlimb defects and mammary gland aplasia, which are similar to those seen in patients with UMS. However, more detailed analysis of *Tbx3*-mutant embryos revealed cardiac anomalies including incomplete looping, OFT defects and malformation of the

AV canal due to altered proliferation rates (532;533). Within the heart, *Tbx3* expression is also required for normal size and function of the sinoatrial (SA) node. Consistent with this, ectopic expression of *Tbx3* is sufficient to induce the pacemaker gene program and function in atrial myocardium (534). A recent analysis of *Tbx3*-null embryos revealed an important role for Tbx3 in the specification of the AV conduction system (139). Bakker M *et al* reported that *Tbx3*-deficient mice have ectopic expression of *Cx40*, *Cx43*, *Nppa*, *Tbx18* and *Tbx20* genes in the AV bundle and proximal bundle branches, which failed to exit the cell cycle. In conclusion, Tbx2 and Tbx3 play important roles in the primary myocardium, where Tbx2 act as a key regulator of the chamber/non-chamber lineage separation upon which all subsequent cardiac morphogenesis depends.

1.4.2.3. Tbx5

The Tbx5 gene was identified in a screen of an E8.5 mouse cDNA library using the T-box region of Tbx2 (535). The mouse Tbx5 gene is expressed in the heart, eye and limb. In the heart, *Tbx5* is expressed in a posterior-to-anterior gradient with relatively high expression in the inflow tract, atrium and left ventricle and low to undetectable levels in the right ventricle. Similarly to Nkx2.5, *Tbx5* is expressed throughout the heart fields early in development. The first line of evidence for a role of Tbx5 in heart development came from its association with Holt-Oram syndrome, a rare highly penetrant dominant condition associated with forelimb and cardiac anomalies (464;465). The role of Tbx5 in heart development was supported by gene targeting experiments in mouse that demonstrated that loss of one *Tbx5* allele recapitulates some of the defects seen in patients with Holt-Oran syndrome. More specifically, *Tbx5*$^{+/-}$ mutant mice have ASD, defects in ventricular relaxation and conduction system abnormalities (119;172;536). Moreover, *Tbx5*-null embryos die at E10.5 and display numerous cardiac anomalies including hypoplastic sinus venosus, failure to initiate atrial septation and dramatic reduction in the expression of chamber specific genes as well as decreased expression of Nkx2.5 and Gata4. Altogether, these studies indicate that Tbx5 plays an important role in chamber specification.

In contrast to *Tbx2* and *Tbx3*, *Tbx5* acts as a transcriptional activator. As discussed in the previous section, a model has been proposed whereby *Tbx5* functions to activate a chamber differentiation program whereas *Tbx2* and *Tbx3* limit the

expression of this program to the ventricle and atrium while repressing it in the conductive tissue (Figure 1.12B). This functions through regulation of the same target genes, which include *Nppa* and *Cx40*. Although *Tbx5* is implicated in several processes during development, only a few target genes were found. However, a few years ago, a microarray analysis of *Tbx5*-induced genes expressed in the heart was reported (537). Among these, in situ hybridization of *photoreceptor cadherin*, *Hey2*, *brain creatine kinase* and *gelsolin* indicated overlapping expression with *Tbx5* in the embryonic mouse heart. In addition, Tbx5 can directly associates with Nkx2.5, Gata4 as well as the transcriptional co-activators Tip60 and Baf60c (119;127;538;539). Acting in synergy with Nkx2.5 and Gata4, Tbx5 can stimulate the expression of chamber specific genes including *Nppa* and *Cx40*.

Tbx5 is also required for the proper development of the cardiac conduction system. Expression of *Tbx5* can be observed in the atrioventricular bundle and the left and right bundle branches in newborn mice. In the *Tbx5*$^{+/-}$ mice, *Tbx5* expression is initiated at the right time in the conduction system but as the mice mature, *Tbx5*-mutant mice develop a prolonged PQ interval, indicative of a first degree AV block (119). Further studies indicated failure of AV node maturation as well as left and right bundle branches defects, including severe reduction in the right ventricular bundle (172). Deficiencies in the downstream target Cx40 did not account for these defects, suggesting that Tbx5 is required in a Cx40-independent pathway in the cardiac conduction system. Moreover, haploinsufficiency of *Tbx5* was shown to cause defects in ventricular relaxation by direct modulation of SERCA2 (536).

To date, about 37 different *Tbx5* mutations have been associated with Holt-Oram syndrome (Figure 1.12C) (481;540). These mutations are clustered near or in the T-box region and may result in truncated or non-functional proteins. Most of these mutations have been suggested to cause haploinsufficiency (541).

1.4.2.4. Tbx20

The *Tbx20* gene was independently identified by several groups in human, mouse and zebrafish models (542-544). Shortly thereafter, orthologs of *Tbx20* were identified in *Xenopus* and chick, where they were also shown to be enriched in the developing heart tissue (545;546). In the mouse, Tbx20 is initially expressed in the primary heart field and a subset of SHF progenitor cells (307;547). In the heart tube,

Tbx20 is detected in the myocardium and also strongly in endocardial cushions of the AVC and OFT (142;307;548). As development proceeds, expression is turned down in the myocardium and remains high in endocardial cushions. A recent series of papers reported that mice lacking *Tbx20* die embryonically at midgestation due to severe cardiac defects including a severely underdeveloped, short heart tube that fails to loop as well as malformation of the ventricular chambers (140-142;307;549). Consistent with a role in the SHF, RNAi mediated knock-down of *Tbx20* in mice was shown to cause hypoplasia of the OFT and right ventricle (307). A key finding in these mutants was that *Tbx2* expression is severely upregulated in *Tbx20*-null embryos. The cardiac anomalies in the *Tbx20* mutants embryos appear to be a requirement for *Tbx20* in the proliferation and maturation of the cardiomyocytes. This seems to be mediated by regulation of *Tbx2* and Tbx20 was found to repress Tbx2 by binding to a pair of T-box binding elements in the Tbx2 promoter (141). Therefore, it has been suggested that one role of Tbx20, maybe to keep Tbx2 off in the developing chamber myocardium. A working model of T-box regulatory network in chamber formation has emerged from all these studies. In the primary myocardium, Tbx2 and Tbx3 compete with Tbx5 to repress chamber differentiation while BMP signalling activates Tbx2, Tbx3 and Tbx20 expression. In the chamber myocardium, Tbx20 represses Tbx2 expression and thus regulates proliferation. This allows Tbx5 to act as a positive regulator of chamber proliferation and differentiation.

Tbx20 is the T-box family member that has the highest expression in endocardial cushions of the AVC and OFT as well as in the remodelling mitral and tricuspid valves. Recent studies provide evidence that Tbx20 promotes proliferation as well as inhibit maturation of valve progenitor cells (550;551). Of note, *Tbx20* gain of function in cultured endocardial cushion cells of chicken embryos results in increased proliferation whereas loss of *Tbx20* leads to a decrease in cell proliferation, with a corresponding effect on N-myc gene expression (550). Moreover, increased Tbx20 expression was associated with an upregulation of matrix metalloproteinases and decreased expression of chondroitin sulphate proteoglycans, which is consistent with the unremodeled state of endocardial cushions.

The role of Tbx20 in the heart appears to be evolutionary conserved, with Drosophila having two *Tbx20* orthologs, *neuromancer 1* (*nmr1*) and *neuromancer 2* (*nmr2*). This pair of genes is expressed in the cardiac lineages, with *nmr1* being the

earliest marker (552;553). Embryos lacking *nmr1* and/or *nmr2* have a weak dorsal vessel phenotype. However, expression of *tinman* (Nkx2.5 ortholog) in the double mutants is very strongly downregulated while expression of the *Tbx2* homologue *Dorsocross* is expanded. More recent studies have demonstrated that adult flies lacking *nmr1* or with reduced *nmr2* expression have compromised cardiac performance (554). Taken together, these results suggest that *nmr1* and *nmr2* are involved in the specification and maturation of the *Drosophila* cardioblasts.

Recently, mutations in human *Tbx20* have been associated with a spectrum of valvuloseptal anomalies; increased cardiac expression of Tbx20 has also been reported in patients with TOF (474;555;556). To date, 12 mutations have been found and 8 of them are within the T-box region, which disrupts the structure and function of the T-box.

In conclusion, Tbx20 acts in the hierarchy of chamber/non-chamber lineage separation to limit the activity of Tbx2. Moreover, the role of Tbx20 in endocardial cells is beginning to emerge and there is much more to be learned in the next couple of years.

1.4.3. MADS transcription factors

The MADS box genes encode a eukaryotic family of transcriptional regulators involved in important biological processes. These proteins share a highly conserved motif called the MADS (MCMI, agamous, Deficiens and serum response factor family) box that mediates homodimerization and DNA-binding to a dyad symmetrical A + T-rich DNA consensus sequences.

1.4.3.1. Serum response factor

Serum response factor (SRF) was first discovered as a factor that bound the serum response element in the c-fos promoter and is the founding member of the MADS family (557). SRF target genes are characterized by the presence of single or multiple copies of the SRF-binding consensus element $CC(A/T)_2A(A/T)_3GG$, known as the CArG box, which are found primarily in genes involved with cell contractility, cell movement and cell growth. SRF expression is largely restricted to the cardiac and skeletal muscle tissues in both chick and mouse development. As cardiac development proceeds, *SRF* transcripts become restricted to the cardiac crescent, the heart tube and the mesenchyme in the tail and somites (538;558). The function of SRF in cardiac

development *in vivo* could not be elucidated due to the early lethality of *SRF*-null mice, which have severe gastrulation defects and die before the onset of cardiogenesis (559). To evaluate the role of SRF in cardiac development, a series of conditional knockout strategy using the $SM22^{cre}$, $MHC\alpha^{cre}$ or $MHC\beta^{cre}$ transgenic mice were performed (560-562). In all studies, cardiac-specific deletion of SRF resulted in embryonic lethality and was associated with cardiac defects including poor trabeculation, disorganization of the cardiac sarcomeres and z-disks, dilated cardiac chambers and thickening of the compact myocardium. In addition, significant reduction of the *ANF* and cardiac *skeletal* and *smooth muscle α-actin* transcripts was observed in *SRF*-mutants embryos. Early inactivation of *SRF* using $Nkx2.5^{Cre}$ transgenic mice results in death by E8-E.5 due to inability of the cardiac cells to start beating (563). Similarly, expression of *Myl2* and *Myom1*, which are expressed in thick filaments and M-band of sarcomeres, *Hand1* and cardiac *skeletal* and *smooth muscle α-actin* transcripts was blocked in *SRF*-mutant embryos. All of these observations indicate that SRF regulates sarcomerogenesis in higher vertebrates.

Although SRF is not considered as an inhibitory transfactor, there is a great likelihood that SRF can exert gene silencing through regulation of miRNAs. Consistent with this, at least 20 miRNAs were found to be downregulated in E9.5 SRF^{Cko} mutants. Among these, Srivastava and colleagues showed that SRF is able to regulate the expression of miR-1, through its CArG boxes (564). Furthermore, mice lacking *miR-1-2* display a spectrum of cardiovascular abnormalities, including VSDs, cardiac rhythm disturbances and hyperplasia of the heart (565). Consistent with a role for miR-1 in muscle differentiation, overexpression of *miR-1* resulted in decreased myocyte proliferation and reduced ventricular myocyte expansion, which was explained by the presence of a miR-1 target site in the 3'-UTR of the *Hand2* gene (564). A few years ago, it was reported that transgenic mice overexpressing SRF develop dilated cardiomyopathy, with early mortality, which suggested that SRF is required in the maintenance of sarcomeric organization and contractility in the adult (566). In addition, it was recently shown that miR-133 can repress myoblast differentiation through repression of SRF (567). Moreover, Olson and colleagues demonstrated that *miR-133a1/miR133a2* compound heterozygotes survive to adulthood and display signs of dilated cardiomyopathy (568). These abnormalities were shown to be attributed, in part, to the increased expression of SRF and Cyclin D2. Thus, a negative feedback loop

exists between SRF and miR-133, where miR-133 is controlled by SRF, which can in turn inhibit SRF translation.

In addition to cardiac sarcomerogenesis, SRF plays an important role in blood vessel development. Of note, specific inactivation of SRF using *Tie2*^{Cre} transgenic mice leads to disruption of the embryonic and yolk sac blood vessels (569). By contrast, the Tie1-mediated loss of SRF resulted in vascular aneurysms and haemorrhaging of the forebrain but no defects were observed in the blood vessels of the yolk sac and vasculature (570). Despite the differences with both strategies, both knockouts resulted in embryonic lethality. Another phenotype that was associated with loss of SRF in endothelial cells was disruption of the actin cytoskeleton in the tip cells, which give rise to sprouting endothelial cells during angiogenesis. Another major type of cell in the vessel wall is the vascular smooth muscle cell (SMC) and expression of SRF in these cells is already evident by E10.5, which coincides with the expression of several SMC contractile genes (538). Specific inactivation of SRF in vascular SMCs results in a significant reduction in the number of peri-vascular progenitor cells as well as a defect in the cyto-architecture of the embryo at E10.5 (560). This cytoskeleton phenotype is similar to that seen in human adult coronary artery SMCs where SRF is knocked down (571). Whether the loss of SRF in vascular SMCs during embryonic development and in the adult has a consequence for normal vascular function still remains to be demonstrated. However, tamoxifen-inducible, smooth muscle *MYH*^{Cre} lines will be useful to inactivate SRF at any time during embryonic development and postnatally in order to have a better understanding of its function in these cells.

1.4.3.2. MEF2

MEF2 proteins belong to the evolutionary conserved MADS family of transcription factors (572). Vertebrates have at least four MEF2-encoding genes whereas simpler animals such as *Drosophila*, *Saccharomyces cerevisiae* and *Caenorhabditis elegans* possess only one *Mef2* gene (573). The N-terminal domain of each MEF2 isoform (Mef2a, Mef2b, Mef2c and Mef2d) contains a highly conserved MADS box and an adjacent motif termed the MEF2 domain, which together mediate DNA binding, dimerization and cofactor interactions (574;575). The C-terminal region functions as a transcriptional activation domain; it is highly divergent among members of the family and also highly variable within a single gene as a result of alternative splicing

(574;576;577). MEF2 proteins bind to the consensus sequence YTA(A/T)4TAR as homo or heterodimers (578;579).

The *Mef2* genes display distinct as well as overlapping patterns of expression in multiple lineages during development including lymphocytes, striated muscles, brain, neural crest, smooth muscle, endothelium and bones (580;581). In the mouse embryo, expression of *Mef2b* and *Mef2c* coincides with the onset of cardiogenesis while expression of *Mef2a* and *Mef2d* starts only a day later (580;582). Inactivation of the *Mef2* gene in *Drosophila* results in failure of differentiation of cardiac, skeletal and visceral mesoderm as well as downregulation of muscle specific gene expression and thus provided the first evidence for the important role of MEF2 proteins in heart development (583-585). Mouse embryos lacking *Mef2c* die at E9.5 due to failure of cardiac looping and inability to form a well developed right ventricle (304). However, cardiomyocytes were still able to differentiate in the absence of Mef2c eventhough a number of cardiac muscle genes were downregulated (304;586). In order to bypass the early lethality of *Mef2c* null embryos, $Mef2c^{loxP/loxP}$ mice were generated to examine the specific role of this protein at later stages of development (587). Inactivation of *Mef2c* using $EIIA^{Cre}$ phenocopied the original *Mef2c* null (587). However, conditional deletion of *Mef2c* with αMHC^{Cre} or $MLC2V^{Cre}$ resulted in viable offsprings with no overt cardiac phenotype, possibly due to functional redundancy with other family members such as *Mef2a*, which is required for maintenance of cardiac physiology after birth. Inactivation of *Mef2a* in mice leads to perinatal lethality due to cardiac defects including severe right ventricular dilatation as well as activation of genes involved in hypertrophy and cardiac failure (588). *Mef2a*-null mice that survived to adulthood showed significant dilated cardiomyopathy, associated with cardiac hypertrophy. In addition, overexpression of either *Mef2a* or *Mef2c* in the adult myocardium leads to dilated cardiomyopathy (589;590). More recently, *Mef2d* emerged as a regulator of stress-dependent cardiac growth and reprogramming of gene expression in the heart (591). Finally, depletion of *Mef2c* in adult myocardium using siRNA attenuates the hypertrophic growth of the left ventricle in response to pressure overload (592).

1.4.4. The GATA transcription factors

The GATA proteins belong to the evolutionary conserved family of zinc finger transcription factors (593;594). Members of the GATA family have been identified in

diverse model organisms ranging from cellular slime moulds to vertebrates including plants, fungi, nematodes, insects and echinoderms (593;595). For example, 29 GATA proteins can be found in *Arabidopsis thaliana*, 11 in *s. cerevisia*, 10 in the nematode *C. elegans*, 8 in *Drosophila melanogaster* and only 6 in humans (594;596). GATA factors are associated with several important processes, including the development of the hematopoietic system as well as cardiovascular development.

1.4.4.1. The GATA factors in invertebrates

GATA transcription factors influence many developmental processes and in addition to vertebrates, a number of GATA proteins have also been identified in non-vertebrates. Fungal GATA factors possess one zinc finger and have been found to play major roles in nitrogen metabolism, light induction, siderophore biosynthesis and mating-type switching (597). For example, in *S. Cerevisiae*, the *Gnl3p* gene binds to GATA elements within the promoters of nitrogen-regulated genes to activate synthesis of glutamine synthase and amino acid permease (598;599).

The *C. elegans* genome has 11 GATA factor genes, and four of these, *end-1*, *end-3*, *elt-2* and *elt-7*, are involved in endoderm development (600;601). *End-1* and *end-3* are the earliest genes expressed in the endoderm lineage and act redundantly in endoderm formation (600;602). Moreover, the *Elt-1* gene is required for epidermal cell fate, *elt-2* is the predominant GATA factor involved in the formation of the intestine and *elt-5* and *elt-6* play a role in epidermal seam cell development (603-605). The *med-1* and *med-2* GATA genes were originally thought to be important for specification of the endoderm but recent studies revealed that this was not the case (606;607). As for *elt-3*, it was suggested to act downstream of *elt-1* in a redundant pathway that controls epidermal cell fate (608).

In *Drosophila*, five GATA genes have been identified and all of them, except for a splicing isoform of *serpent* that lacks the N-terminal zinc finger, have two zing fingers. Interestingly, *Pannier (pnr)* was found to be required for embryonic dorsal closure, dorsoventral patterning of the eye disc and specification of the cardiac lineage (609-612). *Serpent*, on the other hand, plays a role in specification of the hematopoietic lineage, development of fat body and differentiation of the endodermal gut whereas *Grain* is essential for cell rearrangement during organ morphogenesis (613-616). Together, these studies indicate that the function of the GATA factors in endoderm

development between *Drosophila* and *C. elegans* is well conserved. As will be discussed below, some of these functions are also well conserved with vertebrates.

1.4.4.2. The GATA factors in vertebrates

In vertebrates, six members of the GATA family have been identified (GATA1 to -6) and they share two highly conserved zinc fingers, each with a Cys-X_2-Cys-X_{17-20}-Cys-X_2-Cys consensus sequence (Figure 1.13) (617;618). Vertebrate GATA proteins possess two zinc fingers that are encoded by two different exons while only one zinc finger is found in plants and fungi, which is more similar to the C-terminal vertebrate finger. The sequence immediately following the two zinc fingers, known as the nuclear localization domain, is also well conserved in the GATA family. In addition to both zinc fingers, the GATA factors are composed of an N- and C-terminal transactivation domain, which are more divergent (Figure 1.13C). Overall, GATA proteins have a relatively conserved domain structure and yet, are able to achieve diverse roles in a number of cell types. In the following section, a brief description of the structure and role of the zinc fingers will be discussed.

1.4.4.2.1. The structure and role of the C-terminal zinc finger

Several studies have shown that the C-terminal finger and adjacent basic domain of the GATA proteins are required and sufficient for DNA binding (619-621). High resolution nuclear magnetic resonance (NMR) allowed researchers to solve the structure of the binding domain of the chicken GATA1 (cGATA1) C-terminal finger (Figure 1.13B) (622). Omichinski *et al* were able to show that the overall structure of the C-terminal finger domain is composed of two antiparallel β sheets followed by an α helix. The side chains of the zinc finger make specific contacts in the major groove and these interactions are mainly hydrophobic in nature. In addition, the carboxy-terminal basic tail, which is essential for DNA binding specificity, contacts the phosphate backbone in the minor groove. The overall appearance of the complex is analogous to that of a right hand holding a rope, with the palm and fingers representing the protein core, the thumb the carboxyl-terminal tail and the rope the DNA.

The zinc finger regions of the GATA factors are highly conserved, suggesting that they could bind to similar if not identical binding sites (623). The DNA binding affinities and specificities of GATA1, -2, -3 and -6 have been determined using polymerase chain reaction site selection (624-626). GATA1, -2 and -3 can bind to the consensus site

C

GATA-4	TAD	ZF1	ZF2 ++	TAD
GATA-5	49.7 %	87.6 %	++	36.9 %
GATA-6	49.5 %	91.2 %	++	44.2 %

D

Gene	Hematopoietic system	other organs
Gata1	Erythrocytes, megakaryocytes, eosinophils, mast cells and multipotent HSC precusrsors	Sertoli cells
Gata2	Megakaryocytes, mast cells and multipotent HSC precursors	Endothelial cells, inner ear, brain and adipocyte precursors
Gata3	T lymphocytes and NK cells	CNS & PNS, liver, heart, mammary glands, inner ear and adipocyte precursors

E

Gene	Heart	other organs
Gata4	Endocardium, endocardial cushions, myocardium, epicardium	Intestine, bladder, gonads, ovaries, liver, visceral and partietal endoderm
Gata5	Endocardium, endocardial cushions, subset of myocardial cells, epicardium	Instestine, lung, bladder, stomach, urogenital ridge
Gata6	Myocardium, vascular smooth muscle cells	Intestine, lung, bladder

Figure 1. 13. GATA factors in vertebrates

A) Ancestral sequence of the N-terminal finger and C-terminal finger and conservation between the GATA factors (Lowry J *et al*., *Journal of Mol Evol*, 2002). B) Schematic representation of the C-finger of chicken GATA1 bound to DNA (Newton A *et al*., JBC, 2001). C) Protein structure of the cardiac GATA factors and conservation within each domain. TAD: transactivation domain; ZF: zinc finger. D-E) Tables representing the expression profiles of the GATA factors in vertebrates.

derived from regulatory elements in the erythroid cell-specific genes, (A/T)GATA(A/G), with a preference for A/GATA/A. In addition, an alternative consensus DNA sequence, A/GATC/T, is also recognized by GATA2 and -3 but not GATA1 (627). Similarly, Sakai *et al* have showed that the consensus sequence for GATA6 binding is (A/T/C)GAT(A/T)(A). GATA6 also favored adenines on both sides of the core GAT(A/T/C) for strong binding, with a preferential order for GATA>GATT>GATC, with no binding to GATG (626). A novel finding that has emerged from this is the subtle differences in binding specificities that exist among the family members. This raises interesting possibilities on how specific target gene regulation is accomplished in cells expressing more than one GATA protein.

1.4.4.2.2. The role of the N-terminal finger

The two zinc fingers are encoded by two different exons in vertebrate GATA proteins and eventhough the backbone of the N-terminal finger is similar to that of the C-terminal finger, early experiments indicated that it was not essential for DNA binding (628;629). However, more recent experiments have revealed that one of the roles of the N-terminal finger is to enhance DNA-binding specificity and stability of GATA factors (621;628;630;631). According to this, Merika M *et al* showed that several motifs like (C/T/G)GATG(C/G) and (G/C)GATT(C/T) bind with various affinities to intact GATA1 but fail to bind to the C-terminal finger (625). Moreover, Trainor C *et al* found that the N-terminal finger of GATA1 is required to bind with high affinity the sequence ATC(A/T)GATA(A/G), increasing the affinity for this binding site. This palindromic GATA element consist of the consensus sequence (A/T)GATA(A/G) fused to an inverted GAT motif, where the C-terminal finger binds the (A/T)GATA(A/G) sequence whereas the N-terminal finger is involved in binding the partially inverted sequence. In addition to GATA1, other studies have shown that the N-terminal finger of GATA2 and -3 can bind DNA independently, with a slight sequence preference for GATC (632;633).

Another role of the N-terminal finger, and also the C-terminal finger, is the possibility to engage in protein-protein interactions, leading to self-association and binding to other transcription factors. Accordingly, it has been shown that the N-terminal finger of GATA1 can make intermolecular contacts with the C-terminal finger, suggesting that GATA dimers are maintained by direct N-finger-C-finger contacts (634). Importantly, Mackay JP *et al* showed that self-association of GATA1 was disrupted in

the presence of mutations in the N-terminal finger (residues 245-247) and C-terminal finger (residues 315-317) and this also interfered with its ability to activate transcription. The GATA zinc fingers are also able to mediate complexes with FOG, Sp1/EKLF, p300/CBP, c-fos, NFAT-c, SRF, Nkx2.5 and PU.1 (124;367;635-640). FOG-1 (Friend of GATA1) is a 998 amino acid multitype zinc finger protein that was identified in a yeast two-hybrid screen using the N-terminal finger of GATA1 as bait (635). It binds specifically to the N-terminal finger of GATA1 and specific residues located in the amino-finger were eventually identified as essential for this interaction (641). Interestingly, these residues are conserved in the N-terminal finger of all GATA factors known to bind FOG-1. FOG-1 is co-expressed with GATA1 during development and *FOG-1*$^{-/-}$ mice die around E10.5-11.5 due to severe anemia with arrest in erythroid maturation (642). This phenotype is similar to the one that was obtained when the *Gata1* gene was knocked down, providing strong evidence that the two proteins act in a common pathway. A second member of the FOG family, U-shaped, was subsequently identified as a partner for Pannier, a *drosophila* GATA factor (643;644). However, it has been suggested that U-shaped counters the action of Pannier in contract to the cooperative interactions between GATA1 and FOG-1. Soon after the identification of FOG-1, an EST database revealed the existence of a second FOG related gene, known as FOG-2. The expression of FOG-2 mirrors that of Gata4/5/6, suggesting that FOG-2 serves as a cofactor for the cardiac GATA factors. Interestingly, *Fog2*$^{-/-}$ embryos die between E12.5-E15.5 due to complex CHD defects, including overriding aorta, subpulmonic stenosis and subaortic VSDs, as well as impaired coronary vessel formation and gonadal development (645). Similarly to FOG-1, FOG-2 was shown to interact with the N-finger of all GATA factors (646;647). The importance of the physical interaction between GATA4 and FOG-2 was shown by Crispino J and colleagues. A single amino acid replacement in the N-finger of GATA4, which abrogates its interaction with FOG-2, was shown to result in embryonic lethality (489). Mutant embryos displayed defects similar to the *Fog2*$^{-/-}$ embryos as well as additional semilunar valve defects and a DORV. Thus, this study clearly indicated the importance of GATA4/FOG-2 interactions for proper heart development.

1.4.4.2.3. Tissue distribution and function of GATA factors

The six members of the GATA family of transcription factors can be subdivided into two subgroups based on sequence homology and tissue distribution (Figure 1.13D

and E). GATA1, -2 and -3 are highly expressed in the hematopoietic system where they play important roles in lineage specification and differentiation (648;649). GATA1, -2 and -3 are expressed in overlapping subsets of hematopoietic cells as well as in several other tissues. On the other hand, GATA4, -5 and -6, which constitute the second subfamily, are predominantly expressed in the heart and endoderm derivatives such as the gut and lung (650-653). A detailed description of their tissue distribution, function and regulation will be discussed in the following section, with an emphasis on the cardiac GATA factors.

1.4.4.2.3.1. GATA1

1.4.4.2.3.1.1. Tissue distribution and in vivo role of GATA1

GATA1 is the founding member of the GATA transcription family and was originally identified for its ability to bind functionally important DNA regulatory elements found in globin genes (654;655). Expression of GATA1 is restricted to erythrocytes, megakaryocytes, eosinophils, mast cells and multipotential precursor cells within the hematopoietic system (628;655-657). Inactivation of the mouse *Gata1* gene causes embryonic death by E10.5-11.5 due to severe anemia (658;659). Primitive erythroid precursors were not produced in *Gata1*$^{-/-}$ mice and differentiation of erythrocytes and megakaryocytes did not occur (658-660). Likewise, *in vitro* differentiated *GATA1* null ES cells failed to maturate past the proerythroblast stage and undergo rapid apoptosis (618). The murine *Gata1* gene is located on the X chromosome. Takahashi S *et al* demonstrated that male chimeras, which had deletion of the *Gata1* erythroid promoter, die at E12.5 due to an arrest in primitive erythropoiesis, a phenotype that was identical to that observed with *Gata1* null mice (661). Subsequent analysis of hemizygous female embryos revealed that GATA1 is also vital for terminal megakaryocyte differentiation (662). These studies demonstrate an absolute requirement for GATA1 in differentiation of primitive hematopoietic cells.

Outside of the hematopoietic system, the sole site of GATA1 expression is in the Sertoli cells of the testis, starting at postnatal day 7 in mice and lasting during adulthood (663;664). Several studies have shown that gene promoters that are potentially important for Sertoli cell function can be activated by GATA1 (665;666). However, the specific role of GATA1 in Sertoli cells remains elusive.

1.4.4.2.3.1.2. GATA1 in haematological diseases

Since GATA1 has been shown to be essential for hematopoiesis, it is easily acceptable that mutations in the *Gata1* gene could cause haematological diseases. Interestingly, missense mutations in the N-finger of GATA1 have been identified in several families with inherited anemia or thrombocytopenia (667-669). One of these mutations (R216Q) affected DNA binding whereas the other amino acid substitution abolished interactions between GATA1 and FOG-1 (670). Moreover, mutations in the exon 2 (N-terminus) of GATA1 have also been found in neonates with Down syndrome-related acute leukemia (671;672). In this case, an alternative short form of the GATA1 protein, which lacks the first 83 amino acids, is translated from a downstream ATG (Met84) sequence.

1.4.4.2.3.1.3. Regulation of GATA1

GATA1 displays the most restricted pattern of expression compared to GATA2 and GATA3. The *Gata1* gene is transcribed from two different promoters; the most proximal enhancer is responsible for expression of *Gata1* in the hematopoietic cells whereas the most distal promoter directs it expression in the Sertoli cells of the testis (673-675).

In addition, GATA1 can also be modified at the protein level by acetylation, phosphorylation, self-association and interaction with other transcription factors. Six different phosphorylation sites have been mapped to the N-terminal domain of GATA1 and a seventh in the C-terminal zinc finger (676). It was first reported that phosphorylation of GATA1 did not affect its DNA binding affinity but more recent data have suggested otherwise (676;677). The level of GATA1 phosphorylation was shown to increase following induction of erythroid K562 cells, a cell line derived from a patient with myelogenous leukemia, and this increased its affinity for GATA binding elements. An important way by which GATA1 is regulated is acetylation, which stimulates its DNA binding capacity and enhances transcription of target genes (678). Mutation of the main sites of acetylation, which correspond to lysines 246 and 252 in the N-motif and lysine 312 in the C-motif, eliminated the ability of GATA1 to promote erythroid differentiation, suggesting that this modification is essential for its function during hematopoiesis (679). Consistent with this, similar mutations prevented differentiation of 416B myeloid cells to megakaryocytes (620). Recently it was also shown that acetylation and phosphorylation cooperate to trigger degradation of the GATA1 protein

(680). This suggest that the acetylated protein can remain at target promoters until its degradation is activated in response to phosphorylation-mediated signalling. Thus, this is a novel mechanism by which the transcriptionally active GATA1 protein is continuously and carefully regulated during hematopoiesis.

Several studies have demonstrated a role for the N-terminal zinc finger in stabilization of the GATA1/DNA complex as well as influence the specificity of binding. In an attempt to understand the mechanisms that regulate GATA1, Crossley M *et al* found that GATA1 could self-associate through the two zinc finger domains and form protein complexes that synergistically stimulate transcription (681). More detailed studies demonstrated that the two zinc fingers do not homodimerize but rather make intermolecular contacts and that these contacts were greatly reduced when both zinc finger subdomains were mutated (634). Transgenic mice with mutations in the three lysine residues contributing to self-association of GATA1 recapitulated the *Gata1*-null mouse phenotype, providing the first line of evidence that self-association of GATA1 is important for mammalian erythroid development *in vivo* (682). Lastly, protein-protein interactions play significant roles in developmental processes and GATA1, as well as GATA2 and GATA3, have been shown to interact with a number of proteins, including members of the FOG, S1, EKLF and PU.1 families (683).

1.4.4.2.3.2. GATA2

1.4.4.2.3.2.1. Tissue distribution and role of GATA2

In the hematopoietic system, GATA2 is expressed in megakaryocytes, mast cells and hematopoietic progenitor cells, where it overlaps with GATA1 expression, as well as early erythrocytes. Disruption of the *Gata2* locus results in embryonic lethality by E10-11 due to anemia (684). Severe defects in both primitive and definitive erythropoiesis were observed as well as abnormal myelopoiesis and lymphopoiesis, suggesting that GATA2 plays a critical role in the differentiation of early hematopoietic progenitor cells. Further experiments using chimeras generated from *Gata2*$^{-/-}$ ES cells and *in vitro* differentiation of *Gata2* null ES cells revealed a drastic reduction in all hematopoietic compartments, including blood, bone marrow, spleen and thymus. Consistent with this, an *in vitro* differentiation assay using *Gata2* deficient ES cells showed that GATA2 is required more specifically for the proliferation of multipotent hematopoietic progenitors and mast cells, but is dispensable for terminal differentiation

of erythrocytes and macrophages (685). Moreover, enforced expression of GATA2 in primitive hematopoietic cells blocks erythrocyte differentiation (686). These studies all point to an essential role for GATA2 in the proliferation of hematopoietic stem cells and suggest that a decrease in its expression levels is required to induce differentiation. In addition, GATA2 plays a second role during the ontogeny of HSCs, being required for expansion of the HSC population. This was demonstrated by examining the effects of *Gata2* gene dosage on the generation of HSCs throughout mouse development. Ling KW *et al* observed reduced production and expansion of the HSCs in the aorta-gonad-mesonephros region in presence of a haploid dose of GATA2 (687). In contrast, minimal changes were seen in the yolk sac, fetal liver and adult bone marrow.

In addition to the hematopoietic system, GATA2 is expressed in endothelial cells, the inner ear, the brain and adipocyte precursor cells (688-692). GATA2 and GATA3 are both expressed in overlapping regions in the otic epithelium at E10.5 but become more distinct as development progresses (692). Although it was demonstrated that *Gata2* null embryos do not show any defects in the inner ear before death at E10.5, a drastic reduction in *Gata2* transcripts were detected in *Gata3*$^{-/-}$ otic epithelium. These results highly suggested that GATA3 may compensate for the lack of GATA2 in early ear morphogenesis and that both GATA factors act in the same pathway, placing GATA2 downstream of GATA3. More experiments are needed to better understand the function of these proteins in ear development.

A role for GATA2 in fat cell formation has also been proposed. GATA2 and -3 are expressed in murine adipocyte precursors but not in mature adipocytes (691). Continuous expression of these two GATA factors in preadipocytes suppresses terminal differentiation into mature adipocytes. Consistent with this, *Gata3*$^{-/-}$ ES cells were shown to exhibit enhanced capacity to form adipocytes (691). The inhibitory effects of GATA factors on adipogenesis may be mediated, in part, through suppression of the peroxisome proliferator activated receptor gamma (691).

1.4.4.2.3.2.2. GATA2 in haematological disorders

Based on its pattern of expression, the *Gata2* gene is likely to be associated with haematological disorders. However, based on the results of *in vitro* and *in vivo* experiments, it has been hypothesized that reduced expression of GATA2 could lead to aplastic anemia (AA), a bone marrow syndrome characterized by decreased number of

hematopoietic stem cells (693). Consistent with this, different groups found a remarkable reduction of *Gata2* transcripts in patients with AA (694-696). Overall, decreased expression of GATA2 might be responsible for the development of clinical features of the disease.

More recently, mutations in human *Gata2* have been discovered in chronic myeloid leukemia (CML). Of note, in a screen of 85 CML cases, Zhang S *et al* observed a L359V substitution within the second zinc finger of GATA2 as well as an in-frame deletion of 6 amino acids spanning the C-terminal border of the first zinc finger (697). The L359V substitution was associated with increased transactivation activities of GATA2 as well as enhanced downregulation of PU.1 activity, which is required for myeloid cell differentiation. Moreover, when this mutant was introduced into the HL-60 leukemic cell line, it disrupted myleomonocytic proliferation and differentiation *in vitro*, suggesting that GATA2 may play a role in acute myeloid transformation.

1.4.4.2.3.3. GATA3

1.4.4.2.3.3.1. Tissue distribution and function in the hematopoietic system

GATA3 was first described as a transcription factor that binds the TCR-α gene enhancer (698). In the hematopoietic system, expression of GATA3 is confined to T lymphocytes and natural killer cells. Targeted inactivation of *Gata3* in mice leads to embryonic lethality by E11-12 and mutant embryos display severe anomalies including massive internal bleeding, growth retardation, severe deformities of the brain and spinal cord, and defects in fetal liver haematopoiesis (699). *Gata3* null mice also have reduced accumulation of tyrosine hydroxylase (Th) and dopamine beta-hydroxylase (Dbh) mRNA, leading to a reduction in the synthesis of noradrenaline in the sympathetic nervous system (700). Feeding pregnant mice with catechol intermediates partially rescued the embryonic lethality thus indicating that noradrenaline deficiency was the cause of death. The lethal effect of *Gata3* inactivation could be bypassed by using antisense oligonucleotides for GATA3 in fetal thymic cultures (701). In addition, *RAG2*$^{-/-}$ complementation experiments *in vivo* demonstrated that the differentiation of *GATA3* null T cells was blocked at or before the $CD4^-CD8^-$ DN stage of thymocyte development (702). Thus, GATA3 is required for the development of the T cell lineage.

In addition, GATA3 plays a central role in regulating $CD4^+$ T helper 2 (Th2) cell differentiation. Initially, GATA3 was proposed to regulate IFN-γ gene expression in Th1

cells but subsequently, its expression was found to be restricted to Th2 cells (703). Using representational difference analysis, Zheng W and Flavell RA found that GATA3 was upregulated during Th2 cell differentiation. Furthermore, they observed that reducing GATA3 levels in Th2 cells, by using antisense oligonucleotides, prevented the expression of all Th2 cytokine genes. In addition, forced expression of GATA3 was able to repress Th1 development in such a way as to induce Th2 cytokines, namely IL-4, IL-5, IL-13, IL-10 and IL-6 (704). In conclusion, GATA3 is best known to function as a master regulator of Th2-cell differentiation.

1.4.4.2.3.3.2. Functions of GATA3 in organs outside the hematopoietic lineage

In addition to the hematopoietic system, GATA3 is expressed in a variety of tissues including cells of the central and peripheral nervous system, kidney, liver, heart, mammary glands, endothelial cells, ES cells, in the inner ear and adipocyte precursors (692;705-708). A role for GATA3 in mammary glands was also proposed in recent years (709;710). Interestingly, Asselin-Labat ML *et al* showed that lack of *Gata3* in mammary progenitor cells results in expansion of luminal progenitors and concomitant block in differentiation. GATA3 expression was also found to be a marker for well-differentiated tumours in microarray studies of breast cancer (711). In addition, mutations in the *Gata3* gene in humans have also been found in a subset of patients with breast tumours, indicating a potential tumour suppressor role (712).

Recently, a role for GATA3 in heart development was proposed (706). *Gata3* null mice that were treated with sympathomimetic β-adrenergic receptor agonist, which lengthens survival up to E18, developed cardiac anomalies including ventricular septal defects, double outlet right ventricle, persistent truncus arteriosus and aortic arch defects. Interestingly, these defects are similar to those obtained in mice lacking *Gata4* or *Gata6* and in compound *Gata4/Gata6*, *Gata4/Gata5* and *Gata5/Gata6* embryos. A detailed review of the cardiac GATA factors will be given in the following sections and the phenotype of mice haploinsufficient for *Gata4/Gata5* and *Gata5/Gata6* will be discussed extensively in chapter 3 (490). These preliminary results support a role for GATA3 in cardiac development.

1.4.4.2.3.4. GATA4

1.4.4.2.3.4.1. Tissue distribution and in vivo role in embryonic cardiac development

Analysis of regulatory elements in cardiac specific promoters led to the cloning of an additional member of the GATA family, GATA4 (713). GATA4 exhibits a tissue-specific pattern of expression in mice, where it is detected in the heart, gut, gonads, ovaries, liver, visceral endoderm and parietal endoderm (366;652). GATA4 is one of the earliest transcription factors to be expressed in cardiac precursor cells. In the mouse, *Gata4* transcripts can be detected in the precardiac mesoderm at E7.0-7.5 and its expression continues during formation of the heart tube at E8.0, in the endocardium and myocardium (9;366;652;714). As the heart tube elongates and loops (E9.0), GATA4 expression can be found in the sinus venosus, throughout the atrial and ventricular chambers and in the conus (or outflow tract). Between E9.5 and E12.5, abundant GATA4 mRNA can be detected throughout the myocardium, epicardium, endocardial cells and endocardial cushions of the AVC and OFT. This specific expression persists at all other stages of heart development and in the adult.

The first evidence for a role of GATA4 in the heart came from studies in pluripotent P19 embryonic carcinoma cells. Overexpression of GATA4 in these cells increased differentiation of beating cardiomyocytes while its inhibition using antisense RNA prevented cardiomyocyte differentiation and induced massive apoptosis of pre-cardiac cells (715;716). Inactivation of the mouse *Gata4* gene leads to embryonic lethality by E8.0-9.0 due to a failure of ventral morphogenesis and heart tube formation (34;35). Tetraploid complementation was able to rescue the *cardia bifida* phenotype, suggesting that this was due to impaired function of GATA4 in extraembryonic visceral endoderm (717). However, $Gata4^{-/-}$ embryos died around E9.5 and displayed cardiac anomalies including disrupted heart looping, absence of endocardial cushion formation, lack of a proepicardial organ and hypoplastic ventricular myocardium. This early lethality precluded the analysis of the role of GATA4 in later stages of cardiac development, namely during valve formation and maturation of the cardiac chambers.

A series of elegant conditional deletions of the *Gata4* gene have been performed in the last decade to circumvent the early embryonic lethality of *Gata4* null embryos; this tremendously helped to better define the role of this protein during cardiovascular development (Table 1.4). Embryos homozygous for a GATA4 point mutation, which abolishes its interaction with FOG-2, die at E12.5 (489). $Gata4^{Ki}$ embryos had myocardial thinning, common atrioventricular canal, semilunar cardiac valve anomalies and DORV. GATA4 haploinsufficiency in mice causes similar defects, indicating that

Loss of function	*Viability*	*Cardiac Phenotype*	*Reference number*
Gata4 KO	E8.5-E9	*Cardia bifida*	34, 35
Gata4 KO/Tetraploid	E9.5-10	VSD and hypoplasisa	717
$Gata4^{ki/ki}$	E12.5	common AV valve, DORV	489
$Nkx2.5^{Cre};Gata4^{flox/flox}$	E11.5	RV hypoplasia	123
$Tie2^{Cre};Gata4^{flox/flox}$	E12.5	AV cushion defects	719
$\alpha MHC^{Cre};Gata4^{flox/flox}$	E14.5	Myocardial thining and DORV	123
$\alpha MHC^{Cre};Gata4^{flox/flox}$	Viable	Hypoplasia	731
$\beta MHC^{Cre};Gata4^{flox/flox}$	Viable	Hypoplasia	731
$Mef2c^{Cre};Gata4^{flox/flox}$	E13.5	RV hypoplasia and VSD	718
Gata4 hypomorph	E13.5-16.5	Hypoplasia, CAVC and DORV	122
Gata5 KO	Viable	None	757, 758
Gata5 KO	Viable	Bicuspid aortic valve	460
$Tie2^{Cre};Gata5^{flox/flox}$	Viable	Bicuspid aortic valve	460
Gata6 KO	E6.5	Gastrulation defects	773
Gata6 KO/Tetraploid	E9.5-E10.5	None	774
$Wnt1^{Cre};Gata6^{flox/flox}$	E18.5-P1	OFT defects	331
$SM22\alpha^{Cre};Gata6^{flox/flox}$	E18.5-P2	OFT defects	331
$Nkx2.5^{Cre};Gata6^{flox/flox}$	E15.5-E20.5	VSD, irregular septal thickness	775
$\beta MHC^{Cre};Gata6^{flox/flox}$	viable	Mild reduction in heart size	775

Table 1. 4. Cardiac phenotypes associated with loss of function of the GATA factors

cardiac development is sensitive to small changes in GATA4 expression levels. Early deletion of *Gata4* by *Nkx2.5*^{cre} resulted in embryonic lethality by E11.5 with major defects including endocardial cushion defects, right ventricular hypoplasia and myocardial thinning (123). In the same study, the authors reported that late deletion of *Gata4* by *αMHC*^{cre} caused myocardial thinning associated with reduced myocytes proliferation as well as DORV. Specific deletion of *Gata4* in the SHF using *Mef2C*^{Cre} transgenic mice results in lethality by E13.5 with cardiac defects including right ventricular hypoplasia, myocardial thinning, decreased myocytes proliferation and ventricular septal defects (718). Outflow tract defects were not observed using this strategy and previous work has shown that GATA4 is not broadly expressed in the pharyngeal mesoderm (123). Alternatively, the authors suggested that GATA5 and GATA6 could compensate for GATA4 in the SHF since all three cardiac GATA factors are co-expressed in this region. To further investigate the role of GATA4 in endocardial cell development, the *Gata4* locus was mutated by crossing with *Tie2*-Cre mice, which deletes in the endothelial cells (719). By E12.5, 80% of *Gata4*^{T2del} embryos had hypocellular cushions and mutant endothelium failed to undergo EMT, resulting in embryonic death. These results revealed that GATA4 acts to promote cushion mesenchyme growth and remodelling, which is consistent with its expression pattern in endocardial cushions. Overall, these studies clearly demonstrate that GATA4 is required for multiple aspects of cardiac morphogenesis during embryonic development.

1.4.4.2.3.4.2. Role of GATA4 in postnatal heart development

Given the importance of GATA4 in embryonic cardiovascular development and its expression in the adult heart, it is not surprising that this protein also plays a critical function in tissue-specific and inducible gene expression in the adult heart. Indeed, necessary GATA binding elements have been identified within the promoters of most cardiac expressed genes, including atrial natriuretic factor (ANF), b-type natriuretic factor (BNP), α-myosin heavy chain (α-MHC), β-myosin heavy chain (β-MHC), cardiac troponin C and angiotensin type 1 receptor (AT1R) (720-722) (650). Adenoviral mediated transfer of antisense GATA4 into cultured postnatal cardiac myocytes attenuated the expression of several target genes (723). In addition, GATA4 has also been implicated as a critical regulator of inducible gene expression. GATA4 has been shown to respond to hypertrophic stimuli like pressure overload, phenylephrine, endothelin-1 as well as mechanical stretch (721;722;724-727). Other studies revealed

that GATA binding sites were required for the activation of the β-MHC and AT1R following aortic constriction (721;722). GATA sites are also required for *in vivo* response of BNP to angiotensin II (728). A direct role for GATA4 in regulating the hypertrophic response was first described when overexpression of GATA4 in cultured neonatal myocytes or in the heart of transgenic mice induced cardiomyocyte hypertrophy (726). Furthermore, expression of a dominant negative GATA4 engrailed repressor fusion protein or depletion of GATA4 using antisense strategy blocked cystoskeletal organization and features of myocytes hypertrophy in response to PE and ET-1 (726;729). Overall, these studies implicate GATA4 as a necessary and sufficient effector of the hypertrophic response.

Several studies support a role for GATA4 as a critical regulator of cardiomyocyte survival. Inhibition of GATA4 expression in P19 embryonal carcinoma cells blocks expression of downstream target genes as well as triggers massive apoptosis (716). Both *in vitro* and *in vivo* experiments have shown that GATA4 is susceptible to doxorubicin cardiotoxicity, resulting in decreased transcription of target genes in postnatal cardiomyocytes and increased cell death, suggesting that GATA4 is required for myocyte survival (730). Furthermore, deletion of *Gata4* from postnatal and adult cardiomyocytes was shown to promote cardiomyopathy as well as functional decompensation and rapid heart failure following aortic constriction, confirming this hypothesis (731). Lastly, partial *Gata4* deficiency also enhanced cardiac hypertrophy and heart failure associated with cardiomyocyte cells death and increased fibrosis (732). Overall, these studies demonstrate that GATA4 does not only play an important role during cardiac development but is also required in the adult to protect against hypertrophy and for cardiomyocyte survival.

1.4.4.2.3.4.3. Role of GATA4 in other organs

In vivo studies evaluating the role of GATA4 in the developing gonad was not possible until recently because of the early embryonic lethality observed in *Gata4*$^{-/-}$ embryos. Evaluation of gonadal differentiation during embryonic development was made possible by studying *Gata4*^{ki} and *Fog2*$^{-/-}$ animals. *Gata4*^{ki} and *Fog2*$^{-/-}$ male embryos had an absence of testicular cords and failure to express genes required for the onset of testosterone synthesis, Sox9 and MIS (645). These studies showed that in males, interaction between GATA4 and its cofactor FOG-2 are essential for normal

determination and differentiation of the gonads. Moreover, GATA4 was found to differentially activate the expression of steroidogenic enzymes, hormones and transcription factors known to be essential for gonadal development (733).

GATA4 as well as the other two cardiac GATA proteins are expressed in the small intestine in a spatio-temporal manner. Several groups have detected GATA4 protein throughout the small intestine except the distal ileum, where it functions in maintaining jejuna-ileal specific identity (734;735). A key role for GATA4 in the regulation of intestinal gene expression was provided by the observation that expression of the differentiation marker liver fatty acid binding protein is attenuated in *Gata4*-null cells isolated from the small intestine of chimeric mice (736). Consistent with this, specific deletion of *Gata4* in the small intestine, by using two different targeting strategies, resulted in attenuation of enterocyte gene expression from the jejunum but not ileum (737).

In addition, GATA transcription factors are increasingly recognized as playing a role in human cancers. For example, promoter hypermethylation of GATA4 has been reported in human lung, ovarian, gastric, colorectal and esophageal cancer (738-742). Moreover, in colon and gastric cancers, GATA4 target genes, such as *disabled-2* and *inhibinα*, have been found to be epigenetically silenced. The GATA factors have also been associated with pancreatic cancer. Of note, it was observed that GATA4 was upregulated in pancreatic intraepithelial neoplasia, along with other markers such as GATA5, GATA6, Villin1, Villin2, Sox2 and HoxA5 (743). Finally, allelic imbalances in the chromosome locus of GATA4, 8p23.1-p22, is a frequent area of chromosomal imbalance in neoplasms (744). In conclusion, these observations suggest that GATA4 is able to regulate a number of antitumor genes.

1.4.4.2.3.4.4. Regulation of GATA4

GATA4 activity is regulated by numerous posttranslational modifications including phosphorylation, acetylation and sumoylation. Phosphorylation on Ser105 by ERK and p38 MAPK enhances both the DNA-binding and transcriptional activities of GATA4 as well as plays a pivotal role in the hypertrophic response (729;745). A role for ERK1/2 signalling through GATA4 in regulating the hypertrophic response was suggested by the observation that dominant negative GATA4-engrailed expressing adenovirus attenuated MEK1-induced cardiac hypertrophy (745). While both ERK and p38MAPK

increase the potency of GATA4, glycogen synthase kinase 3β (GSK3β) negatively regulate its activity. Phosphorylation of the N-terminal transactivation domain of GATA4 by GSK3β was shown to increase the nuclear export of GATA4 and this effect was reversed when the cells were treated with leptomycin B (746). In addition, *in vitro* phosphorylation via protein kinase A on serine 261 leads to the recruitment of CBP (747). More recent studies have demonstrated that GATA4 is a direct target of protein kinase C in cardiomyocytes, which phosphorylates GATA4 within its C-terminal domain (748). This results in enhanced DNA binding and transcriptional activity where GATA4 physically interacts with STAT1 to activate angiotensin II and other growth factor inducible promoters.

Besides phosphorylation, GATA4 activity can also be influenced by acetylation. Several studies have suggested that GATA4 may be acetylated in cardiomyocytes during the hypertrophic growth response, and event that seems to be mediated by p300. Of note, a dominant-negative form of p300 was shown to inhibit agonist-induced hypertrophy as well as GATA4 dependent transcriptional activity in cardiomyocytes (749). The authors also observed that transgenic mice with cardiac specific overexpression of p300 were able to promote myocardial hypertrophy, which was associated with increased GATA4 acetylation and DNA binding activity.

Finally, the transcriptional activity of GATA4 is also affected by sumoylation from small ubiquitin-like modifiers (SUMO). Interestingly, GATA4 was shown to be sumoylated by SUMO1 on lysine 366, which resulted in enhanced transcriptional activity (750). When this lysine was mutated, there was less GATA4 protein in the nucleus, suggesting that SUMO modification also modulates GATA4 nuclear localization.

Overall, these modifications are important as they affect the DNA binding activity as well as transcriptional activity of GATA4, which plays critical roles during embryonic development. Thus, mutation of one residue that abrogates either acetylation, phosphorylation or sumoylation can have devastating consequences during cardiac development as it will prevent interactions of GATA4 with cardiac cofactors and reduce target gene activation.

1.4.4.2.3.4.5. GATA4 in congenital heart disease

The human *Gata4* gene maps to chromosome 8p22-23. The first evidence of a role for GATA4 in CHD was suggested when deletion of the distal arm of the chromosome 8p was found in heterozygous individuals with septal and valve defects (751). Subsequently, missense and nonsense *Gata4* mutations, G296S and E359del, were identified in familial cases of ASDs (127). The G296S mutation decreased GATA4 DNA-binding affinity and transcriptional activity whereas the frameshift mutation impaired transcriptional activity. Additional heterozygous GATA4 mutations have since been identified in patients with different forms of CHDs (470;752;753). Of note, Nemer G *et al* reported the presence of a missense mutation, E216D, in patients with tetralogy of Fallot that resulted in decreased transcriptional activity of GATA4 (754). To date, these studies have suggested that the underlying mechanism of pathogenesis is haploinsufficiency.

1.4.4.2.3.5. GATA5

1.4.4.2.3.5.1. Tissue distribution and in vivo role in cardiac development

GATA5 was first identified in *Xenopus* and chicken by low stringency hybridization to the GATA1 DNA binding domain and was later found to be expressed in the heart and gut (652;755). Expression of GATA5 is restricted both spatially and temporally in embryonic and postnatal development. The GATA5 protein is expressed in the heart, the lung bud, the allantois, urogenital ridge, bladder and gut epithelium during embryonic development and in the bladder, lung, stomach and small intestine in the adult (366;652;756). In the heart, GATA5 transcripts are initially detected in the precardiac mesoderm at E7-7.5 and subsequently in the primary heart tube at E8.0. However, between E9.5 and E12.5, its expression becomes more restricted to the endocardial cells and endocardial cushions of the AVC and OFT. Shortly thereafter (E16.5), GATA5 transcripts can be no longer detected within the heart. In addition, GATA5 mRNA and protein can also be detected in the epicardium, which is thought to contribute to interstitial cardiac fibroblasts, coronary smooth muscle cells and endothelial cells (383).

The dynamic expression of GATA5 in endocardial cells suggests a specialized role for this transcription factor in endocardial development. Consistent with this, mutation of the *Gata5* gene, encoded in the *faust* locus, in zebrafish causes embryonic lethality and results in *cardia bifida*, similar to the phenotype observed in *Gata4* null

mice. *faust* mutants had a significant loss of myocardium and showed a loss in the expression of several myocardial genes including Nkx2.5 and Gata4 (33). In addition, loss of function studies in an *in vitro* model of endocardial differentiation abolishes expression of terminal differentiation markers and inhibit endocardial cell differentiation, suggesting an important role for GATA5 in endocardial differentiation (367). However, targeted inactivation of *Gata5* in mice did not produce a detectable cardiac phenotype; null mice were viable and fertile but female *Gata5*$^{-/-}$ mice displayed genitourinary tract abnormalities (Table 1.4) (757;758). Given the prominent roles of GATA5 in the heart in the zebrafish and other model systems, this was a surprising result and it raised the possibility that GATA4 could compensate for GATA5 function in the heart. Alternatively, the strategy used, which targeted the first exon may have produced a null allele. Indeed, it was demonstrated that two different *Gata5* transcripts could be produced, one that corresponded to the full length GATA5 protein and one that lacked the entire exon 2 but contained the DNA binding zinc finger and the C-terminal activation domain (759;760). Further evidence from our lab demonstrated that this N-terminal truncated protein, which lacks the N-terminal finger, retains its ability to bind DNA and activate target genes (365). So, the presence of a possible truncated protein cannot be ruled out.

As describe in this thesis, we have produced a *Gata5* null mouse line and we show that loss of function of *Gata5* in mice leads to bicuspid aortic valve formation, which is consistent with the expression of GATA5 in endocardial cells and endocardial cushions of the OFT (Table 1.4). Our strategy targeted exons 3 to 6, which codes for the C-terminal zinc finger and the whole C-terminal region, therefore ensuring that both isoforms would be knocked down.

1.4.4.2.3.5.2. Role of GATA5 in other organs

A key role for GATA5 in endoderm development has been described in *Xenopus*, where ectopic expression of GATA5 re-specifies ectodermal and mesodermal cells towards an endodermal fate (761). This function in endoderm development seems to be conserved in zebrafish. Defective formation of endodermal derived organs, including the liver, pancreas, thymus and thyroid, was observed in the *faust* mutants in addition to the *cardia bifida* phenotype (33;762). GATA5 expression is also observed in the endoderm in avians and mammals but, in contrast, deletion of the *Gata5* locus in

the mouse had no consequence on endoderm development, suggesting that this function is not conserved in mammals (384;756;763).

Consistent with the expression of GATA5 in the urogenital ridge during development, it was shown that female *Gata5* null mice exhibit pronounced anomalies of the genitourinary tract including vaginal and uterine defects and hypospadias (757). However, a direct role in bladder, intestine, stomach or lung development has not been reported yet.

There is growing evidence to link the loss of *Gata5* gene function, and also the other cardiac GATA factors, with malignancy of various organs. In this regard, diminished GATA4/-5 expression has been reported in ovarian, gastric, colorectal and lung cancers (738;739;741;764). In addition, the chromosome regions of GATA4 and GATA5 are frequent targets of deletion in neoplasms (765). Epigenetic silencing of GATA5 by aberrant methylation has also been reported in primary gastrointestinal and lung cancers (738;739). Together, these observations suggest a role for GATA5 in cell differentiation.

1.4.4.2.3.5.3. Regulation of GATA5

Enhancers that govern *Gata5* gene expression have only been reported in chicken so far. Two promoters differentially regulate GATA5: the proximal promoter directs *Gata5* gene expression within a subset of mesodermal cardiac cells early in heart development whereas the more distal enhancer is responsible for expression in the endoderm (759;766;767). Although the proximal enhancer is initially expressed in the cardiac crescent, heart expression becomes confined to a subset of myocardial cells in the common atrial chambers by E8.5 and in the epicardium and endocardial cushions of the AVC by E9.5.

In addition to promoter regulatory elements located in the *Gata5* locus, the use of an alternative first codon can also regulate GATA5 activity. Of note, the gene encoding chicken and mouse GATA5 possesses two alternative non-coding exons that produce two different proteins by differential splicing of the respective transcripts (759) (760). Interestingly, the truncated GATA5 protein is composed of the C-terminal zinc finger and C-terminal transactivation domain and retains the ability to transactivate target promoters albeit with less efficiency than full length GATA5. These findings

suggest that the expression and function of GATA5 might be more complex than previously appreciated.

1.4.4.2.3.6. GATA6

1.4.4.2.3.6.1. Tissue distribution and specific role in cardiac development

The GATA6 protein can be detected in the heart, lungs, urogenital ridge, bladder and epithelium of the gut during embryonic development and in the gastrointestinal tract, bladder, lungs and the heart in the adult (366;756). In the heart, *Gata6* transcripts are first detected in the precardiac mesoderm at E7.0-7.5, where they co-localize with GATA4 and GATA5. Up to E9.5, the expression of GATA6 is almost identical to that of GATA4 within the developing heart, with the highest levels detected in the posterior regions. By E11.5, *Gata6* mRNA can be detected in the myocardium of the developing atria and ventricles, the endocardium and in the outflow tract, albeit at lower levels than GATA4. In contrast to GATA4, *Gata6* transcripts are observed in the caval veins and dorsal aorta between E12.5 and E13.5, with expression confined to vascular smooth muscles (768-770). Cardiac expression of GATA6 persists in later stages of cardiac development and in the adult.

In *Xenopus*, expression of GATA6 can first be detected at the beginning of gastrulation in the mesoderm and cardiac progenitors (771). GATA6 expression decreases in heart precursors as the cardiac gene transcription machinery commences. Elevating GATA6 expression beyond this time in cardiac cells delays the onset of cardiomyocyte differentiation, suggesting that GATA6 maintains the heart cells in a precursor state, needing to be downregulated for heart cells to mature. Loss of function in *Xenopus* and zebrafish, using antisense morpholino oligonucleotides, results in heartless embryos with a concomitant drastic reduction in *Bmp-4* and *Nkx2.5* gene expression (772). This supports a role for GATA6 in the maturation of the cardiac progenitors rather than their initial induction. These studies provided the first evidence for a potential role of GATA6 in heart development. Targeted inactivation of *Gata6* in mice leads to embryonic lethality shortly after implantation (E6.5) owing to defects in extraembryonic endoderm differentiation, precluding assessment of the function of GATA6 in cardiovascular development (Table 1.4) (773). Analysis of rescued *Gata6*$^{-/-}$ embryos revealed no major heart phenotype, suggesting that this GATA factor was dispensable for early heart formation in mice (774). However, it was recently shown

that specific deletion of *Gata6* in the myocardium, using the *Nkx2.5*^{Cre} transgenic mice, leads to embryonic lethality between E15.5-E20.5 due to irregular septal thickness and ventricular septal defects (775).

GATA6 is abundantly expressed in vascular smooth muscle cells (VSMC) during mouse embryonic and postnatal development (768). To better determine the role of GATA6 in VSMCs, conditional inactivation of GATA6 was achieved using the SM22α promoter, which is expressed in neural crest derived SMC, mesoderm-derived SMC and in cardiac myocytes. Mutant embryos died perinataly from cardiac anomalies including a spectrum of aortic arch patterning and cardiac OFT septation defects. An identical phenotype was observed when GATA6 was specifically inactivated with *Wnt1*-Cre reporter mice in neural crest cells. The phenotype of interrupted aortic arch and PTA, a defect in which the truncus arteriosus fails to separate into an aorta and pulmonary artery before birth, is recapitulated in *semaphorin 3C* null mice, a signalling molecule critical for vascular patterning (332;776). Interestingly, *Gata6* mutant embryos showed reduced expression of semaphoring 3C. These results support a role for GATA6 in the regulation of aortic arch patterning and cardiac outflow tract septation, at least in part through activation of semaphoring 3C gene expression.

Mice lacking one copy of each *Gata4* and *Gata6* are embryonic lethal and show myocardial thinning and outflow tract defects (777). This suggests that the two GATA factors can partially compensate for each other, and that a threshold of GATA4 and GATA6 is required for normal heart development. These results may also reflect interactions of these two GATA factors, for activation of common target genes. Consistent with this, mice lacking both GATA factors do not develop hearts but have normal expression of cardiac progenitor cell markers in the cardiac crescent, suggesting that GATA4 and -6 have essential roles in ensuring that the progenitors follow a cardiac myocytes fate during heart development (778).

Recently, mutations in GATA6 have been associated with CHDs, including tetralogy of Fallot, PTA and ASDs (473;779;780). Three different mutations were located around the same cluster in the N-terminal domain of the GATA6 protein, another one in the second zinc finger and the fifth one in the NLS. One of these mutations, A178V, was associated with increased transcriptional activity of mutant GATA6 (780). The GATA6-E486 del mutant created a truncated protein that lacked the

C-terminal transactivation domain (779). Moreover, this mutation abrogated the transcriptional activity of GATA6 on downstream target genes SEMA3C and plexin2A, which are required in the development of the OFT.

1.4.4.2.3.6.2. Role of GATA6 in the liver and lungs

In addition to the heart, the GATA6 protein is highly expressed in the liver, where it was shown to regulate the *Hnf4* gene (781). Moreover, development of the *Gata6* null embryos arrest during gastrulation with a phenotype that is very similar to that of the *Hnf4*$^{-/-}$ embryos (773;781;782). A role for GATA6 in liver formation was demonstrated by rescuing the *Gata6* null embryos with wildtype extraembryonic visceral endoderm (774). Rescued *Gata6*$^{-/-}$ embryos survived up to E10.5 and analysis of the phenotype revealed that hepatic cells failed to differentiate and the liver bud did not expand. The authors suggested that the *Gata6* phenotype might result from functional redundancy of GATA4 and GATA6 at the early stages of hepatic specification. Consistent with this hypothesis, injection of morpholinos that inhibit both GATA4 and GATA6 completely eliminates the liver bud and prevents liver-specific gene expression (783).

In addition to the liver, *Gata6* mRNA is expressed in the fetal pulmonary epithelium (784). Loss of function experiments, using antisense oligonucleotides and *Gata6*$^{-/-}$ chimeric lungs, results in branching morphogenesis and epithelial cell differentiation defects. In addition, overexpression of GATA6 in transgenic mice using a pulmonary epithelium specific promoter causes a very similar phenotype, likely suggesting that proper levels of GATA6 are required for terminal differentiation of the pulmonary epithelium (785).

1.4.4.2.3.6.3. Regulation of GATA6

Original description of the cardiac GATA proteins suggested high similarity of their overall structure. However, it has been established that in mouse, human, *Xenopus* and zebrafish GATA6 can exist as a longer polypeptide through usage of an alternative translation initiation codon (769;772;786). This new GATA6 isoform contains an additional 146 amino acids in the N-terminal domain, is abundant in several tissues including the heart and has greater transcriptional activation potential.

In avians and mammals, three distinct enhancer regions that affect cardiac expression have been identified in the *Gata6* locus (787-789). In both species, the

GATA6 cardiac enhancer contained a binding site for Nkx2.5 that was essential for expression in cardiogenic cells. A second enhancer was found to direct *Gata6* gene expression to the atrioventricular conduction system; a more distal enhancer region can affect some expression in the gut (167;790;791).

Overall, the available evidence support and essential role for all three cardiac GATA factors in heart development. Moreover, these GATA factors interact together for some aspects of morphological events during heart development in diverse model organisms. Combinatorial interactions between these factors and with other cardiac transcription factors is of critical importance as a mutation that impairs interaction between cofactors can have devastating consequences during embryonic cardiac development, which may subsequently result in CHDs.

Hypothesis

GATA5, a member of the GATA family of transcription factors, is expressed in a spatial and temporal manner in the developing heart where it is predominantly found in endocardial cells and ECs of the OFT and atrioventricular canal between E9.5-E12.5 in the mouse. Previous studies have established that *Gata5* plays a critical role in differentiation of endocardial cells. However, targeted deletion of *Gata5* in mice did not produce a detectable phenotype, which could be attributed to the production of a shorter GATA5 isoform, which lacked the N-terminal. Based on these observations, we generated a new *Gata5* null allele, deleting the exons encoding the second zinc finger and whole C-terminus, to evaluate its role during cardiac morphogenesis. I have shown that *Gata5* null mice develop bicuspid aortic valves, with a penetrance of 26%. As the aortic valve is formed from the endocardial cushions, we were also interested to find what cell type was responsible for this defect. To that end, we generated a *Gata5* specific deletion in endothelial cells and again, this resulted in formation of BAVs, with a ratio of 21%.

During heart development, the expression of the three cardiac GATA factors partially overlaps and it has also been shown that they can bind similar DNA regulatory elements and activate common target promoters. Moreover, it was previously demonstrated that GATA4 and GATA6 genetically interact together during heart development in mice, *Xenopus* and zebrafish. Based on these observations, we were interested to find out if GATA5 could genetically interact with GATA4 and GATA6 in cardiac morphogenesis. To test this possibility, we decided to generate mice double heterozygous for *Gata5* and/or *Gata4* and *Gata6*. I have showed that both sets of compound heterozygotes die perinataly and exhibit cardiac defects including double outlet right ventricle and VSDs. These results, coupled with those of the *Gata5* null mice, highly suggest that human GATA5 may be a potential candidate congenital heart disease causing gene. Finally, this raises the possibility that subtle alterations in the level or activity of 2 cardiac GATA factors might lead to congenital heart disease in human.

Chapter 2

Loss of GATA5 in mice leads to bicuspid aortic valve

Short title: Essential role of *Gata5* in heart development

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2.1. Abstract

Bicuspid Aortic valve (BAV) is the leading congenital heart disease and occurs in 1-2% of the population. Genetic studies suggest that BAV is an autosomal dominant disease with reduced penetrance. So far only one gene, *Notch1* has been linked to some BAV. Here, we show that targeted deletion of *Gata5* in mice leads to hypoplastic hearts as well as partially penetrant BAV formation. Similar to *Gata5* null mice, endocardial specific inactivation of *Gata5* leads to BAV. In all cases, the observed BAVs resulted from fusion of the right-coronary and non-coronary leaflets, the subtype associated with the more severe valve dysfunction in human. Neither endocardial cell proliferation nor cushion formation was altered in the absence of GATA5. Rather, defective endocardial cell differentiation resulting from deregulation of several components of the Notch pathway as well as other important endocardial cell regulators may be the underlying mechanism of disease. The results unravel a critical cell autonomous role for endocardial GATA5 in aortic valve formation and identify GATA5 as a potential congenital heart disease causing gene in human. Mice with mutated *Gata5* alleles represent unique models to dissect the mechanisms underlying degenerative aortic valve disease and to develop much needed preventive and therapeutic interventions.

2.2. Introduction

Proper formation and function of the heart valves is critical for unidirectional blood flow within the four chambered mammalian heart and valve dysfunction leads to serious cardiovascular complications. Valve disease, whether congenital or acquired, is a major clinical problem worldwide and valve replacement is the second leading cardiac surgery in North America.

Bicuspid aortic valve (BAV) is the most common congenital cardiac malformation and occurs in 1-2% of the population (1). It is generally diagnosed in adulthood when deterioration of the abnormal leaflets becomes clinically evident with affected individuals developing valve disease 10 years earlier than those with normal aortic valve leaflets. Patients with BAV are at increased risks of developing serious complications, including aortic stenosis, aortic regurgitation and endocarditis; one third of these patients will in fact develop significant cardiovascular complications and many will require surgical interventions. Population studies have suggested that BAV may be responsible for more mortality and morbidity than all other congenital heart defects (CHD) combined (2). Despite this, our understanding of the mechanisms underlying BAV formation remains limited. BAV occur either in isolation or in association with other malformations such as coarctation of the aorta, ventricular septal defects and hypoplastic left ventricle (3-5). Genetic studies have established that BAV is a highly heritable trait with an autosomal dominant transmission and incomplete penetrance (6;7). So far, only one gene, *Notch1*, has been linked to BAV in human with mutations found in some but not all BAVs (8;9). Genome-wide scans have suggested linkages to several human chromosomal regions but no other disease causing genes have yet been identified (10). In animal models, BAVs have been found in a subset of mice lacking endocardial nitric oxide synthase (*NOS3*) or the cardiac transcription factor Nkx2.5 but neither gene has been associated yet with human BAV (11;12). Better knowledge of the molecular pathways governing valve development may help identify BAV causing genes.

Over the past years, molecular and genetic analysis of heart development have started to identify genes and pathways involved at various stages of valvulogenesis (13). Valve development is a complex process that involves expansion and differentiation of endocardial cells, their migration following en endocardial-mesenchymal transformation

(EMT) to form endocardial cushions at the atrioventricular (AVC) canal and within the outflow tract (OFT). The AV cushions then give rise to the mitral and tricuspid valves whereas the aortic and pulmonary valves arise from the OFT. In addition to endocardial cells, other cell types participate in valve development, notably neural crest and secondary heart field derived cells, which play important roles in the formation of the OFT. Bone morphogenetic proteins BMP2 and 4 are critical myocardial derived signals that modulate the EMT. BMP and BMP-regulated transcription factors can promote both proliferation and differentiation of EC cells and are part of a complex regulatory network that must tightly regulate cell-cell interactions and cell fate throughout valvulogenesis. Mice lacking components of the BMP pathway or transcription factors that control the proliferation and differentiation of endocardial cells, such as Twist1, Msx, or the Notch target Slug1, have defective EMT. Similarly, proper levels of vascular endothelial growth factor (VEGF) are needed to promote proliferation and survival of endocardial cells prior to EMT (14;15).

Another critical pathway for valve development is Notch, which plays multiple roles throughout valvulogenesis. Notch1, 2 and 4 and the Notch ligands DLL4 and JAG1 are expressed in endocardial cells where they regulate endocardial differentiation and EMT. The importance of this pathway is underscored by the finding that mutations of *Notch1* are linked with human BAV and that mutations in *JAG1* and *Notch2* cause Alagille syndrome, an autosomal dominant disease that affects the cardiac OFT (16-18). JAG1 mutations have also been identified in other forms of CHD with EC involvement (19;20). Notch proteins are cell surface receptors that are cleaved by γ-secretase complex upon ligand binding, releasing the Notch intracellular domain, which then translocates to the nucleus, associates with RBP-Jκ to switch it from a transcriptional repressor to an activator. Notch target genes include transcription factors as well as signaling molecules such as neuregulin, VE-cadherin and the bHLH proteins Hey/Hrt, which regulate BMP signaling. Consistent with a critical role for the Notch pathway in endocardial cushion development, inactivation of several pathway components in mice produce cardiac defects (21). Endocardial Notch signaling is also required for endocardial-myocardial interactions, specifically in ventricular trabeculation, and may potentially explain the link between BAV and hypoplastic left ventricles (22).

Other families of transcription factors are present in endocardial cells and regulate endocardial differentiation and valve formation. They include the forkhead

proteins FoxP1 and Foxc2 as well as the SOX proteins Sox4 and 9 (23-25). Tbox proteins have also emerged as important regulators of valvulogenesis; Tbx2 and Tbx3 are required for establishing the AV boundary while Tbx20 promotes endocardial cell expansion and differentiation (26-28). Lastly, genetic and biochemical studies have suggested important roles for members of the GATA family of zinc finger proteins in endocardial cell expansion and differentiation. Tissue specific deletion of *Gata4* in endothelial cells causes embryonic lethality by E12.5 because of defects in epithelial-mesenchymal transformation, resulting in the formation of hypocellular endocardial cushion (29). In humans, mutations in GATA4 have been found in association with septal defects (30-32). More recently, mutations in another GATA gene, GATA6, which in the heart is expressed predominantly in myocytes as well as neural crest derived cells, have been reported in human CHD (33). In contrast to GATA4, which is expressed in both myocardial and endocardial cells, GATA5 expression in the heart is largely restricted to endocardial cells where it is transiently expressed during embryonic development (34;35). *In vitro* studies revealed a requirement for GATA5 for differentiation of committed cardiogenic precursors into endothelial endocardial cells (36). In zebrafish, *faust* (which encodes Gata5) mutants lack endocardial cells and have a reduced number of myocytes (37).

In this study, we show that targeted inactivation of the *Gata5* gene in mice affects heart development and leads to bicuspid aortic valve. Deletion of GATA5 specifically from endocardial cells is sufficient to recapitulate the cardiac phenotype of *Gata5* null mice, suggesting a cell autonomous function of GATA5 in regulating endocardial cushion differentiation. Mechanistically, we found that GATA5 regulates several pathways associated with endocardial cell differentiation, including BMP4, Tbx20, NOS3 and Notch. Together, the data reveal an important function for GATA5 in aortic valve development and identify GATA5 as an important regulator of mammalian heart development and a candidate CHD causing gene.

2.3. Results

Gata5 null mice have mild left ventricular hypertrophy

The mouse *Gata5* gene contains 6 exons and spans 10 Kbp of DNA. We generated the targeted allele by introducing LoxP sites flanking exon 3 and exon 6 through homologous recombination in ES cells (Figure 1A). These exons encode the second zinc finger essential for DNA binding, the nuclear localization sequence and the complete C-terminus. The presence of the targeted allele in ES cells was confirmed by southern blot (Figure 1B). PCR analysis confirmed the presence of the WT (448 bp) or Floxed (285 bp) alleles (Figure 1C). Mice heterozygous for *Gata5*$^{neo\text{-}LoxP}$ were bred to CMV-cre females, which deletes in the germline, resulting in *Gata5*$^{+/-}$ mice. *Gata5*$^{+/-}$ mice were intercrossed to generate *Gata5*$^{-/-}$ mice on a 129/C57BL/6 mixed genetic background. Homozygous *Gata5* mice were viable and obtained at the expected Mendelian ratios. Q-PCR analysis at E12.5 confirmed that exons 3-6 had been deleted in *Gata5* null mice (Figure 1D). Due to the presence of the first coding exon in the *Gata5* targeted allele, a truncated protein containing the N-terminal portion of GATA5 could still be produced. However, Q-PCR analysis indicated a 90% reduction in transcripts from the first two coding exons in *Gata5*$^{-/-}$ mice, suggesting that no GATA5 protein is likely to be produced as a consequence of nonsense mediated mRNA decay (Figure 1D).

Anatomical examination of *Gata5*$^{-/-}$ mice revealed a cardiac phenotype; at first sight, right atrial enlargement was visible and heart size appeared mildly increased (Figure 2A-D). Echocardiography as well as measurement of heart weight and ventricular mass confirmed that *Gata5*$^{-/-}$ hearts were larger than control littermates. Echocardiography performed on sex matched *Gata5*$^{-/-}$ and wild-type controls at 70 days (n = 11-14 per group) revealed increased thickness of the interventricular septum (IVS) (0.643 mm ±0.028 vs 0.814 mm ±0.030, p=0.002), left ventricular posterior wall (LVPW) (0.658 mm ±0.021 vs 0.772 mm ±0.014, p<0.001) and increased left ventricular mass (3.840 mg/g ±0.168 vs 4.524 mg/g ±0.183, p<0.05) in *Gata5*$^{-/-}$ hearts (Figure 2G-I). Identical results were also obtained at 180 days (data not shown). The ejection fraction, a measure of left ventricle performance, was slightly, but consistently higher in *Gata5*$^{-/-}$ hearts (57.752 % ±2.061 vs 66.570 % ±2.271, p=0.019), suggesting a hypercontractile state (Figure 2J). Histological analysis and myocyte counts indicated that the increased

mass is due to myocyte enlargement not hyperplasia (Figure 2E, F). In fact, *Gata5* null ventricles had fewer myocytes per field than control littermates (19±2.5 vs 28±0.88; p=0.03). Increased *ANF*, *BNP* and *skeletal actin* mRNA levels were observed as early as 30 days in the LV and IVS of *Gata5*$^{-/-}$ mice (*n* = 6-8 per group) consistent with the presence of left ventricular hypertrophy (Figure 2K, L and data not shown).

Dysregulated cardiac morphogenesis and bicuspid aortic valve in *Gata5* null mice

Since GATA5 is highly expressed in endocardial cushions of both the outflow tract and atrioventricular canal we checked whether its deletion disrupts valve formation or function. Hemodynamic evaluation of *Gata5*$^{-/-}$ mice at 70 days showed increased velocity and pressure gradients at the level of the mitral, aortic and pulmonary valves, suggestive of valve disease, which could contribute to development of ventricular hypertrophy (Figure 3A, B; data not shown). Analysis of the aortic root area revealed a significant decrease in *Gata5* null mice relative to WT littermates (n=11-13 per group) indicative of mild aortic stenosis; this was evident as early as 70d (1.26 mm^2 ±0.05 vs 1.13 mm^2 ±0.05, p<0.05) and was further accentuated in older 180d old mice (1.51 mm^2 ±0.05 vs 1.34 mm^2 ±0.04, p<0.05). Furthermore, a subgroup (22%) of *Gata5* null mice had a much higher velocity (1061.757 mm/s ±35.480 vs 1906 mm/s ±110.620, p<0.01) and mean gradient (4.569 mmHg ±0.313 vs 14.653 mmHg ±1.704, p=0.015) through the aortic valve (3/14) (Figure 3A, B). Morphologic examination of the valves revealed the presence of bicuspid aortic valves (BAVs) in 25% (7/28) of *Gata5* homozygous mice as compared to 3% (1/29) in the control group (Figure 3C-G). No other structural abnormalities were evident at the level of the other valves or the septa. Thus, GATA5 seems to be essential for normal aortic valve development.

To determine whether the postnatal hypertrophy of *Gata5* null mice is present in embryonic hearts or whether it reflects a compensatory mechanism, the cardiac phenotype of *Gata5*$^{-/-}$ embryos was carefully analyzed. At E11.5, both the LV and RV walls were thinner in *Gata5*$^{-/-}$ embryos compared to control littermates (Figure 4A-F). Moreover *Gata5*$^{-/-}$ hearts were hypotrabeculated as compared to their controls. To determine if these changes were due to reduced number of myocytes and/or endocardial cells, we counted both cell types using the Image J software. Significant decrease in the number of myocytes (328.497 ±18.565 vs 244.667 ±20.701, p=0.011)

but not endocardial cells was evident in the LV of *Gata5* null embryos (Figure 4G, H). Thus, during development, lack of GATA5 results in hypoplastic hearts, which likely undergo compensatory hypertrophy postnatally.

The valve leaflets of the heart and interventricular septum (IVS) originate from the endocardial cushions (ECs), where GATA5 expression is enriched. Cushion formation is localized in the outflow tract (OFT), where the pulmonary and aortic valve will form, and in the atrioventricular canal (AVC), which is responsible for mitral and tricuspid valve formation. We found that the number of mesenchymal cells was reduced in both the AVC (345.867 ±20.066 vs 272.667 ±15.059, p=0.021) and OFT (338.333 ±15.542 vs 258.000 ±17.387, p=0.032), raising the possibility of reduced survival, proliferation or migration of mesenchymal cells within the endocardial cushions (Figure 4I, J). TUNEL assays and phosphohistone H3 immunostaining were carried out on E11.5 tissue sections to measure cell apoptosis and proliferation. No significant changes, in either the AVC or OFT, were detected between *Gata5* null and control embryos. Therefore, morphogenesis of the OFT cushions was analyzed in more details. The OFT cushions were formed properly in *Gata5* null embryos with reduced number of mesenchymal cells (Figure 4K, L). The septal ridge was abnormally fused with the posterior intercalated cushion, leading to the fusion of the right and non-coronary valve leaflets (R-N BAV), which in humans is associated with a greater degree of complications compared to other BAV subform (38). Next, we verified whether formation of the cardiac jelly, which is critical for EC development, is altered in *Gata5* null mice. The cardiac jelly results from transformation of a subset of endothelial cells in the endocardium into mesenchymal cells that will migrate and invade the extracellular matrix through a process known as endothelial-mesenchymal transformation (EMT). Sections of WT and *Gata5*$^{-/-}$ embryos at E11.5 were stained with alcian blue, which stains acid glycosaminoglycans that marks the EMT (Figure 4M, N). Alcian blue staining was detected in both controls and *Gata5* null mice and there were no major differences between the two genotypes, suggesting that GATA5 is not required for cardiac jelly formation. This hypothesis was further supported by the finding that transcripts for *Has-2*, the major component of the cardiac jelly, remained unaltered in *Gata5* null hearts at E12.5 (data not shown). Together, these results suggest that GATA5 may regulate genes involved in endocardial cell migration and/or differentiation.

GATA5 regulates the Notch pathway

Gene expression patterns in embryonic and postnatal hearts of *Gata5* null and control mice were analyzed using Q-PCR. As shown on Figure 6, expression of the two other cardiac GATA factors, GATA4 and 6, were unchanged in *Gata5*$^{-/-}$ embryonic or adult hearts (Figure 5A, B and data not shown). This was confirmed by immunohistochemistry where intact levels of GATA4 were observed in control and *Gata5* null embryos (Figure 5T, U). However, a significant reduction in the mRNA levels of other transcription factors including *Tbx20* (47%), *Mef2c* (34%) and *Bmp4* (25%) was observed in *Gata5* null hearts (Figure 5C, D, E). Reduction of *Tbx20* was confirmed in endocardial cells by immunohistochemistry (Figure 5V, W).

As mentioned earlier, Notch signaling is critical for proper cardiovascular development and mutations in *Notch1*, *Notch2* or *Jag1* (a Notch ligand) have been associated with OFT defects in humans (8;18;39). Moreover, *Jag1* mutations have also been associated with Tetralogy of Fallot and pulmonary stenosis (19;40;41). We analyzed expression of various Notch components; at E12.5, the mRNA levels of *Notch1*, *Notch2*, *Notch4* and *Dll4* were similar in *Gata5* null and control embryos (Figure 5 and data not shown). However, a 35% decrease in *Jag-1* transcripts was observed in *Gata5* null embryos (Figure 5H). Moreover, a 2-fold increase in the mRNA of the Notch transcriptional effector *RBP-Jκ* was observed (Figure 5G). Given that in the absence of Notch activation, RBP-Jκ acts as a transcriptional repressor, the finding that Jag1 expression is decreased while that of RBP-Jκ is increased suggests dysregulation of the Notch pathway in *Gata5* null hearts. Consistent with this, we observed decreased immunostaining for the Notch1 intracellular domain (NICD) and Jag1 as early as E10.5, confirming that the Notch pathway is downregulated in *Gata5* null mice (Figure 5P-S). Accordingly, a significant decrease in the Notch targets *Neuregulin-1* (30%) and *Hey-1* (20%) was found (Figure 5I, N). Other endothelial and endocardial cell markers like *VE-cadherin* (20%), *Tie-2* (20%) and *EphB4* (25%) were also downregulated (Figure 5K-M).

Endothelial nitric oxide synthase (NOS3) plays an important role in aortic valve formation as shown by the presence of partially penetrant R-N BAVs in *NOS3* null mice, the subform of defects seen in *Gata5* null mice (12;38). NOS3 expression was downregulated as early as E10.5 in the left ventricle and the outflow tract of *Gata5*$^{-/-}$ embryos compared to their wild-type controls (Figure 6 A-D). Bioinformatic analysis of

the murine *NOS3* promoter revealed three evolutionary conserved GATA binding sites (Figure 6E). GATA5 was able to bind to these three GATA elements with high affinity (Figure 6F). Additionally, in cotransfection experiments, GATA5 enhanced *NOS3* promoter activity to a greater extent than GATA4 (18 vs 5-fold activation) (Figure 6G). The results identify that *NOS3* as a GATA5 target; reduction in NOS3 may be a contributing mechanism to BAVs.

Endocardial GATA5 is required for aortic valve formation

Formation of the OFT cushion is accompanied by migration of mesenchymal cells from the neural crest, the pharyngeal mesenchyme and endocardium derived mesenchyme. To determine which cell type is responsible for the formation of BAVs in *Gata5* null mice, we mutated the *Gata5* gene specifically in endothelial cells by crossing with *Tie2-cre* transgenic mice to obtain *Tie2-cre;Gata5*$^{Flox/Flox}$ mice. This approach was selected because GATA5 is enriched in endocardial cells but absent from vascular endothelial and neural crest cells (35;36). In mice carrying the *Tie2-cre* transgene, recombination occurs as early as E9.5 in ECs of both the OFT and AVC that will eventually give rise to the semilunar (pulmonary and aortic) and atrioventricular (mitral and tricuspid) valves (42). *Tie2-cre;Gata5*$^{Flox/Flox}$ mice were obtained at the expected mendelian ratios and were viable. As in *Gata5* null mice, we found BAVs in 21% (3/14) of *Tie2-cre;Gata5*$^{Flox/Flox}$ mice compared to 1% (1/31) in control *Tie2-cre;Gata5*$^{+/+}$ mice littermates (Figure 7C-E). Close examination of the morphology of the OFT cushions of *Tie2-cre;Gata5*$^{Flox/Flox}$ embryos at E11.5 revealed identical results to those of *Gata5* null embryos, namely abnormal fusion between the posterior intercalated cushion and the septal ridge, creating an R-N BAV (Figure 7F, G).

Q-PCR analysis for exons 4-6 in E12.5 *Tie2-cre;Gata5*$^{Flox/Flox}$ embryos confirmed strong downregulation of *Gata5* transcripts with low residual expression (Figure 8A). At the cell level, immunostaining with the anti-Gata5 antibody indicated that *Gata5* expression was significantly reduced in most endocardial cells as early as E10.5 (Figure 8G-J). No significant change in *Gata4* and *Gata6* transcript levels was noted in *Tie2-cre;Gata5*$^{Flox/Flox}$ embryos (Figure 8B, C). However, a strong downregulation of *Tbx20* (50%) and *Jag-1* (50%) transcripts and a significant decrease in *erbB2* (30%) mRNA was detected in these hearts (Figure 8D-F). Immunostaining confirmed that Jag1 expression was downregulated in *Tie2-Cre+;Gata5*$^{Flox/Flox}$ embryos at E10.5

(Figure 8M-N). In addition, reduced expression of NICD was noted in these embryos indicative of defective Notch pathway.

Altogether, these results indicate that GATA5 is an important regulator of genes involved in endocardial cell differentiation and that expression of GATA5 in endocardial cells is required for proper development of the endocardial cushions. Moreover, the data reveal that absence of GATA5 results in defective valve morphogenesis and BAV formation.

2.4. Discussion

In this study, we used mouse genetics to determine the function of GATA5 in mammalian embryogenesis. The results reveal an essential role for GATA5 in heart morphogenesis and a critical cell-autonomous role in endocardial cushion formation and aortic valve development. In particular, we show that deletion of the *Gata5* gene results in BAV formation.

BAV is the most common congenital heart malformation and occurs at a rate of 1-2% of the population. Although BAVs can function normally, the valve leaflets are subject to increased haemodynamic stress, which can lead to serious complications including aortic stenosis, aortic regurgitation, incompetence and calcification. It is also estimated that over half of valve replacements and incidence of patients with coarctation of the aorta can be attributed to BAV disease. In fact, BAVs have been associated with greater morbidity and mortality than all the other CHDs combined. In humans, most BAVs result from fusion of either the right and left leaflets (R-L) or the right and non-coronary leaflet (R-N). The R-N BAV is associated with greater degree of valve dysfunction and it has also become clear over the years that BAV morphology is of prognostic relevance in the management of patients with BAVs (43-45). Work in animal models suggests that the 2 subtypes may be distinct etiological entities; the R-N BAVs would result from defective development of the cardiac OFT endocardial cushions while R-L BAVs result from an extra fusion of the septal and parietal ridges (38). The BAVs in *Gata5* null mice are in all cases the result of a fusion between the posterior intercalated cushion and the septal ridge, giving rise to the R-N subtype. The differential formation of R-N type BAVs supports the hypothesis that the different BAV subtypes have distinct genetic etiologies. As a corollary, differential outcomes in humans with R-N and R-L BAVs may be due to distinct underlying genetic pathways.

Cardiac valves are derived from the endocardial cushions, which are rich in extracellular matrix components. Defective development of the heart valves occurs in 20-30% of all CHDs (46;47). There is increasing evidence that loss of extracellular matrix (ECM) organization is associated with changes in mechanical properties, leading to dysfunction in adult valve disease. A number of studies have shown that periostin is required for normal cardiac valve development and maturation (48;49). Valve leaflets of *periostin*$^{-/-}$ mice are hypertrophied and shortened by 3-months of age and the tendinous

cords of the AV valves are either truncated or missing. The phenotype of *periostin*$^{-/-}$ mice is similar to the degenerative changes seen in prolapsed human mitral valves or BAVs. Periostin levels are also pathologically overexpressed in infiltrated inflammatory cells and myofibroblasts in areas of angiogenesis in human atherosclerosis and rheumatic valve disease (50). Versican, another EMC component, plays important roles during cardiac development and in adult cardiovascular diseases (51;52). Versican cleavage occurs throughout cardiac development by members of the ADAMTS family such as, *Adamts9* which is expressed in mesenchymal cells of the valves. *Adamst9* haploinsufficiency leads to abnormal thickening of the semilunar valve leaflets as well as increased proteoglycan content in the aortic valve (53). Proper elastic fiber assembly and function are critical for aortic valve and aortic wall integrity. Mutations in elastins, fibulin family members and other components of elastic fiber assembly result in progressive adult disease in animal models and in human including supravalvar stenosis (54;55). No significant changes in mRNA levels for periostin, elastin and several other ECM relevant genes were found in *Gata5*$^{-/-}$ embryonic hearts. Whether changes develop in aging *Gata5* null mice deserves to be assessed.

Valve development requires complex interactions between transcription factors that regulate proliferation, differentiation and leaflet remodeling. GATA5 appears to regulate at least two pathways involved in differentiation of endocardial cells, namely Tbx20 and Notch. Tbx20 is a member of the *T-box* gene family that is expressed in myocardial as well as endocardial cells during avian and mammalian development. Deletion of *Tbx20* in mice results in embryonic lethality, reduced myocardial differentiation and defective chamber maturation (56). Knockdown of *Tbx20* with siRNAs provided the first evidence for Tbx20 involvement in valve development (57). More recently, two studies showed that Tbx20 was required for proliferation and migration of mesenchymal cells within the cushions (28;58). Importantly, mutations in the *Tbx20* gene have been linked with valve and septal defects in humans (59;60). Our results show a 45% reduction of *Tbx20* transcripts in *Gata5* null and *Tie2*-cre+;*Gata5*$^{Flox/Flox}$ embryos along with reduced number of mesenchymal cells in both the AVC and OFT cushions at E12.5. Thus, Tbx20 may be a downstream target of GATA5 in endocardial cell. Another critical regulator of valvulogenesis is the Notch pathway. Mutations in the *Notch1* gene have been associated with BAV in humans and mutations in *JAG1* and *Notch2* have been associated with Alagille syndrome, which is

characterized by multiple outflow tract defects (8;18;39). GATA5 and JAG1 are both expressed in endothelial and endocardial cushion cells of the OFT and AVC at E12.5 and our study reveals a significant downregulation of *Jag-1* transcripts in *Gata5* null and *Tie2*-cre+;*Gata5*$^{Flox/Flox}$ embryos with concomitant upregulation of the transcriptional repressor RBP-Jκ (36;61). Decreased ligand level together with upregulation of the transcriptional repressor would be expected to result in significant attenuation of functional Notch pathway in endocardial cells. In this respect, it is noteworthy that loss of Notch1 or RBP-Jκ from endocardial and endothelial cells was shown to result in hypotrabeculated, hypoplastic hearts (22). Our results are in line with this study and support a regulatory function for endocardial Notch signaling in myocardial morphogenesis.

Lastly, the data presented in this manuscript confirm a role for GATA5 in endocardial cell differentiation *in vivo*, which is consistent with our previous *in vitro* work and with the phenotype of the zebrafish *faust* mutant, which encodes GATA5 (36;37). However, the phenotype of our *Gata5* null mice differ from that of a previously described *Gata5*$^{-/-}$ line that displayed no overt cardiac phenotype (62). The *Gata5* locus produces two protein isoforms through alternative translation initiation; in addition to the translation start site in exon 1, use of a second ATG upstream of exon 2 generates a truncated GATA5 protein comprising aa 225-404, which retains one zinc finger, DNA binding and transcriptional activation properties (63;64). In contrast to the targeting strategy used by Molkentin *et al*., which only deletes one isoform, our strategy was designed to delete exons 3-6, which contain the DNA binding, nuclear localization and C-terminal transactivation domains therefore ensuring that both isoforms are eliminated. While this work was in progress, another group reported the production of mice carrying a *Gata5*^{tm2Eem} mutated allele that deletes both zinc fingers coding exons (65). The *Gata5*^{tm2Eem} mice did not display an apparent cardiac phenotype but showed a 2-fold overexpression of both GATA4 and GATA6 mRNA. Crossing these into a *Gata4*$^{+/-}$ background produced hypoplastic ventricles and severe endocardial cushion defects a phenotype that resembles that observed in our *Gata5* null mice. This raises the possibility that upregulation of the other cardiac GATA factor may have compensated for lack of GATA5. In our *Gata5* null hearts, we did not detect any changes in GATA4 or GATA6 levels in embryonic or postnatal hearts. Moreover, female *Gata5*$^{-/-}$ mice did not show a reduction in the distance between the vagina and anus as observed in the other

Gata5 mutated alleles. At present, the reasons for the differential phenotype remain unclear but genetic background contribution to the manifestation of congenital heart disease is well documented (31). Be it as it may, the results of the present study suggest that GATA5 activity within the proximal and distal OFT is important for the development of the aortic valve and document for the first time the role of endocardial cells in the pathogenesis of BAVs.

GATA5 is broadly but transiently expressed in endocardial and endocardial cushion cells of the AVC and OFT (35;36). The *Gata5* null mice did not display detectable defects in other endocardially derived structures such as the atrial septum or other valves. Our results suggest that the number of endocardial cells and endothelial-mesenchymal transformation was not altered in the absence of GATA5; rather it appears that differentiation and possibly migration or cell-cell interactions are disrupted. It is possible that GATA4 may be able to partially compensate for GATA5 in earlier stages of endocardial cell expansion and differentiation; alternatively GATA5 window of expression during development may affect only a subgroup of genes and processes involved in OFT development such as interactions with the secondary heart field or neural crest derived cells. Either way, the finding that loss of GATA5 from endocardial cells differentially affects aortic valve leaflets will help in furthering our limited understanding of how common endocardial cushions contribute to specific valve leaflets.

In conclusion, the data presented here are consistent with a crucial role for GATA5 in aortic valve formation and suggest that GATA5 may be a disease causing gene. Future studies aimed at elucidating the upstream regulators and downstream targets of GATA5 in endocardial cells will contribute to mechanistic understanding of endocardial cushion development as well as gene pathways involved in BAV and other valve disease.

2.5. Methods

Animals

Mice handling and experimentation were performed in accordance with institutional guidelines. All protocols were approved by the institutional Animal Care Committee.

Histology

Adult tissues or staged mouse embryos at E10.5 and E11.5 were fixed in 4% paraformaldehyde, paraffin embeded, sectioned at 4-um intervals and processed as previously described. Anti-Gata4, Gata5 and anti-Tbx20 antibodies were previously described (35;57;66). Polyclonal anti-eNOS, Notch1 NICD and Jag1 were purchased from Abcam (catalog numbers, respectively, ab66127, ab8925 and ab7771). The biotinylated anti-rabbit IgG antibody was purchased from Vector Laboratories (BA1000).

Cell count

Image J software was used to count the number of myocytes, endocardial cells and cushions cells in three different sections of 3-4 different heart samples for each genotype.

Gel shift assay

Nuclear extracts of NIH 3T3 overexpressing GATA5 were obtained as previously described (35). The probe used for GATA binding corresponded to the -265 GATA element (5'- GTTCCCACTTATCAGCTCTAGCCC-3').

Generation of GATA5 mutant mice

The 5′-arm, KO arm (exons 3-6) and 3'-arm of the *Gata5* genomic locus were isolated from the bMQ221g13 BAC clone (67). A 5.9-Kb genomic DNA fragment (5'-arm) that included exons 1 and 2 with flanking introns was digested with Avr2 restriction enzyme and subcloned upstream of the LoxP site of the targeting vector. A 3.8-Kb fragment (the deleted region) that encoded exons 3-6 was digested with Pac1 and Nhe1 restriction enzymes and inserted in between the LoxP sites. A 4-Kb fragment (3′-arm) was digested with Sal1 and Kpn1 restriction enzymes and subcloned downstream of the LoxP site in the targeting vector.

The targeting vector was digested with Not1 and Kpn1 and electroporated into 129Sv embryonic stem (ES) cells. Following negative selection with G-418, 400 individual ES cell clones were isolated and analyzed for homologous recombination by southern blotting. Two clones with a properly targeted *Gata5* allele were microinjected into 3.5-d C57BL/6 blastocysts, which were implanted into CD1 pseudopregnant females. The resulting chimeras were bred to C57BL/6 mice to achieve germline transmission. To obtain mice with a *Gata5*-null allele, $Gata5^{WT/\text{neo-loxP}}$ mice were crossed with CMV-Cre mice to generate $Gata5^{+/-}$ mice, which were then intercrossed together to obtain $Gata5^{-/-}$ mice. The *Gata5* null mice were maintained in a mixed 129SV/C57BL/6 background and analyzed between generations F3 to F5.

A similar breeding strategy was used to generate mutant mice lacking Gata5 in the endocardium. Of note, $Gata5^{WT/\text{neo-loxP}}$ mice were crossed with *ACTB1-Flpe* mice to generate $Gata5^{+/\text{loxP}}$ mice, which were then crossed with *Tie2-cre* transgenic mice to obtain $Tie2\text{-}cre;Gata5^{+/Flox}$ mice. $Tie2\text{-}cre;Gata5^{+/Flox}$ mice were then intercrossed together to obtain $Tie2\text{-}cre;Gata5^{+/+}$, $Tie2\text{-}cre;Gata5^{+/Flox}$ and $Tie2\text{-}cre;Gata5^{Flox/Flox}$ mice. $Tie2\text{-}cre;Gata5^{Flox/Flox}$ mice were kept in a mixed 129SV/C57BL/6 background and analyzed between generations F2 to F5.

Tail genomic DNA was digested with Sca1 or Drd1 and analyzed using a standard Southern blot protocol with the probes indicated in Figure 1.

Bicuspid and tricuspid aortic valve analysis

Hearts were perfused with 4% paraformaldehyde in PBS and then fixed overnight at 4ºC. Atria were removed under the microscope and the aortic arch and pulmonary artery were cut at an angle to reveal the aortic valve.

Echocardiography

Transthoracic echocardiography was performed using a visual sonics Vevo 770 ultrasound system with a RMV 707 30 MHz transducer as previously described (66). Doppler and M-mode imaging was obtained from 70 days and 225 days old mice. Statistical analysis was done using Student's two-tailed *t*-test. Groups of 11-14 mice from different litters were used for the M-mode measurements and Doppler readings at 70 days while groups of 5-6 mice were used at 225 days.

Quantitative polymerase chain reaction (Q-PCR)

Total RNA was isolated from hearts of E12.5 embryos or from left ventricular and interventricular septum at 30 days postnatal with TRIZOL reagent (Invitrogen); cDNAs were generated using the Omniscript RT kit (Qiagen) and QPCR was performed as previously described (68). Primers sequence is available on request.

Statistical analysis

Values are presented as mean ±S.E.M. and *n* refers to the number of mice per group. *P* values were generated using the Student's two-tailed *t*-test, and statistical significance was considered as $P<0.05$.

2.6. Acknowledgements

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2.8. Figure legends

Figure 2.1. Generation of a *Gata5* null allele. (**A**) Schematic representation of the *Gata5* locus and targeting strategy. Positions of the 5′-probe and 3′-probe used for Southern blots are shown. Cre-mediated excision removes exons 3-6, leaving one LoxP site. Coding exons are in pink and non-coding exons are in light-grey. TAD, transactivation domain; ZF, zinc finger. (**B**) Southern blot analysis of targeted ES cells. Genomic DNA was digested with Sca1 and hybridized to the 5′-probe on the left panel. On the right panel, genomic DNA was digested with Drd1 and hybridized to a 3′-probe. (**C**) Genotyping of wild-type (+/+), heterozygous (+/-) and homozygous (-/-) targeted allele. PCR using primers a-b and a-c identifies product corresponding to wild-type (448bp) and knockout (285bp) alleles. (**D**) Q-PCR analysis of *Gata5* transcripts in hearts of wild-type, *Gata5*$^{+/-}$ and *Gata5*$^{-/-}$ embryos at E12.5. Results show complete reduction of Gata5 exons 4-6 (dark grey) and exons 1-2 (light grey) expression in *Gata5*$^{-/-}$ mice. GAPDH was used as an internal control (** P<0.01).

Figure 2.2. Mild left ventricular hypertrophy of *Gata5*$^{-/-}$ mice. (**A-D**) Anatomical analysis of wild-type (**A**, **C**) and *Gata5*$^{-/-}$ (**B**, **D**) mice. Frontal view orientation (**A, B**) of the hearts showing mild increase in heart size of *Gata5*$^{-/-}$ (**B**) mice. Trichrome staining of *Gata5*$^{+/+}$ (**C**) and *Gata5*$^{-/-}$ (**D**) hearts. Note the increased heart size, right atrial enlargement and increased left ventricular internal dimension of *Gata5*$^{-/-}$ mice. Bars = 1500 µm. (**E, F**) High magnification of cardiomyocytes showing increased cell size in *Gata5*$^{-/-}$ mice. Bars = 20 µm. (**G-I**) Echocardiography of control (*Gata5*$^{+/+}$) and *Gata5*$^{-/-}$ mice of 70 days (* P<0.5, ** P<0.01, *** P<0.001, n = 11-13 per group). Note the increased thickness of the interventricular septum (IVS), left ventricular posterior wall (LVPW) and left ventricular mass (LV mass), suggesting the presence of left ventricular hypertrophy in *Gata5*$^{-/-}$ mice. (**J**) Echocardiography of wild-type and *Gata5*$^{-/-}$ mice at 70 days showing an increase in the ejection fraction of *Gata5*$^{-/-}$ mice (* P<0.5, n = 11-13 per group). (**K**, **L**) Enhanced atrial natriuretic factor (ANF) and brain natriuretic peptide (BNP) expression in *Gata5*$^{-/-}$ left ventricle (LV) and interventricular septum (IVS) as revealed by Q-PCR analysis of wild-type and *Gata5*$^{-/-}$ mice at 30 days (* P<0,05, ** P<0.01, n = 6-8 per group).

Figure 2.3. Valvular dysfunction of *Gata5*$^{-/-}$ mice. (**A-B**) Echocardiography of wild-type and *Gata5*$^{-/-}$ mice at 70 days showing increased mean velocity and pressure gradients

through the aortic valve (AV) (* P<0.05, ** P<0.01, n = 11-13 per group). (**C**, **D**) Anatomical analysis of *Gata5*$^{-/-}$ mice revealing the presence of bicuspid aortic valve and tricuspid aortic valve. Arrows indicate the point of attachment of the valve cups to the aortic wall. Bars = 500μm. (**E**, **F**) Trichrome staining of the aortic valve of *Gata5*$^{-/-}$ mice showing the presence of two or three leaflets. Stars indicate the number of leaflets. Bars = 500μm. (**G**) Summary of the number of *Gata5*$^{+/+}$ and *Gata5*$^{-/-}$ mice with BAVs. The table shows that 25% of *Gata5*$^{-/-}$ mice have BAVs compared to control littermates.

Figure 2.4. Reduced trabeculation and R-N type of BAV in *Gata5* null embryos. (**A-F**) Trichrome staining of transverse sections of *Gata5*$^{+/+}$ and *Gata5*$^{-/-}$ hearts at E11.5. *Gata5*$^{-/-}$ embryos have a thinner left ventricle and right ventricle and are less trabeculated than the controls. Bars = 300μm (**A**, **D**) and 50μm (**B**, **C**, **E**, **F**). (**G-J**) Quantitation of the number of cells in *Gata5*$^{-/-}$ embryos compared to control littermates at E11.5 (* P<0.05, n = 3-5 per group). Note the reduction of the number of myocytes within the left ventricle (LV) while the number of endocardial cells remains unchanged. The numbers of mesenchymal cells in the AVC and OFT of *Gata5*$^{-/-}$ embryos is also significantly decreased by 20%. AVC, atrioventricular canal; EC, endocardial cell; LV, left ventricle; OFT, outflow tract; RV, right ventricle. (**K**, **L**) Trichrome staining of transverse sections of OFT of *Gata5* null embryos at E11.5. The arrow points to the abnormal fusion of the posterior intercalated cushion with the septal ridge, creating a bicuspid aortic valve of the R-N subtype. AVC, atrioventricular canal; SR, septal ridge; P, posterior intercalated cushion; PR, parietal ridge. Bars = 75 μm. (**M**, **N**) Alcian blue staining was used to visualize acid glycosaminoglycans, such as hyularonic acid, within the endocardial cushions of the OFT. *Gata5* null embryos had a similar amount of alcian blue staining in the OFT endocardial cushions at E11.5. Bars = 75 μm.

Figure 2.5. Modulation of gene expression in *Gata5*$^{-/-}$ embryos. (**A**, **B**) Q-PCR analysis showing normal levels of *Gata4* and *Gata6* in the hearts of *Gata5*$^{-/-}$ embryos at E12.5 (n = 6-8 per group). (**C-E**) Q-PCR analysis showing altered expression of *Tbx20*, *Bmp4* and *Mef2c* in hearts of *Gata5*$^{-/-}$ embryos at E12.5 (* P<0.05, n = 6-8 per group). (**F-J**) Q-PCR analysis of members of the Notch pathway. Expression of *Notch1* and Hey-2 remains stable in *Gata5*$^{-/-}$ embryos at E12.5 (n = 6-8 per group). A significant upregulation of *Rbp-jκ* transcripts is observed. Also note the downregulation of *Jag-1* and *Hey-1* transcripts in *Gata5*$^{-/-}$ embryos at E12.5 (* P<0.05, n = 6-8 per group). (**K-O**) Q-PCR analysis showing altered expression of several endothelial markers in *Gata5*$^{-/-}$

embryos at E12.5 (* P<0.05, n = 6-8 per group). (**P-W**) Transverse sections of E10.5 control and *Gata5*$^{-/-}$ embryos stained for NICD (**P**, **Q**), Jag1 (**R**, **S**), Gata4 (**T**, **U**) and Tbx20 (**V**, **W**). Note the decreased NICD,Jag1 and Tbx20 expression in the *Gata5*$^{-/-}$ embryos (**Q**, **S**, **W**). Bars = 40 µm.

Figure 2.6. GATA5 regulates *NOS3* expression. (**A**, **B**) Transverse section of E10.5 *Gata5*$^{+/+}$ and *Gata5*$^{-/-}$ embryos showing reduced NOS3 expression in endocardial cells of the left ventricle of *Gata5*$^{-/-}$ embryos. Bars = 30 µm. (**C**, **D**) Transverse outflow tract section of E11.5 *Gata5*$^{+/+}$ and *Gata5*$^{-/-}$ embryos showing reduced NOS3 expression in *Gata5*$^{-/-}$ embryos. Bars = 75 µm. (**E**) Schematic representation of the murine *NOS3* promoter with the conserved GATA binding sites (top). (**F**) DNA binding of GATA5 expressing NIH 3T3 cells on the proximal GATA binding element of the *NOS3* promoter. Note how binding is displaced by a GATA5 specific antibody (Ab) or by addition of excess cold probe (self, G2, G3). (**G**) Fold activation of the -1.6Kbp and -265bp *NOS3* promoter by increasing amounts of GATA4 and GATA5 in NIH 3T3 cells. The data is the average of a duplicate experiment repeated three times.

Figure 2.7. *Gata5* is required in endocardial cells for aortic valve formation. (**A**, **B**) Trichrome staining of *Tie2*-cre+;*Gata5*$^{+/+}$ and *Tie2*-cre+;*Gata5*$^{Flox/Flox}$ frontal sections. There are no major differences in heart size or wall thickness between the two groups. (**C**, **D**) Anatomical analysis of *Tie2-cre;Gata5*$^{Flox/Flox}$ mice revealing the presence of bicuspid aortic valve. Arrows indicate the point of attachment of the valve cups to the aortic wall. Bars = 400 µm. (**E**) Summary of the number of *Tie2-cre;Gata5*$^{+/+}$ and *Tie2-cre;Gata5*$^{Flox/Flox}$ mice with BAVs. The table shows that 21% of *Tie2-cre;Gata5*$^{Flox/Flox}$ mice have BAVs compared to control littermates. BAV, bicuspid aortic valve. (**F**, **G**) Trichrome staining of transverse sections of OFT of *Tie2-cre;Gata5*$^{+/+}$ and *Tie2*-cre+;*Gata5*$^{Flox/Flox}$ embryos at E11.5. The arrow points to the abnormal fusion of the posterior intercalated cushion with the septal ridge, creating a bicuspid aortic valve of the R-N subtype. SR, septal ridge; P, posterior intercalated cushion; PR, parietal ridge. Bars = 75 µm.

Figure 2.8. Modulation of gene expression in *Tie2-cre;Gata5*$^{Flox/Flox}$ embryos. (**A-C**) Q-PCR analysis showing strong reduction of *Gata5* transcripts in *Tie2-cre+;Gata5*$^{+/+}$ and*Tie2-cre+;Gata5*$^{Flox/Flox}$ embryos at E12.5 (* P<0.001, n = 6-7 per group). Also note that the expression of *Gata-4* and *Gata-6* mRNAs remains normal in *Tie2-*

cre;Gata5$^{Flox/Flox}$ embryos. (**D-F**) Q-PCR analysis showing strong reduction of *Tbx20* and *Jag-1* transcripts in *Tie2*-cre+;*Gata5*$^{Flox/Flox}$ embryos at E12.5. Also note the significant downregulation of *erbB2* transcripts (* $P<0.05$, n = 6-7 per group). (**G-J**) GATA5 immunostaining of E11.5 transverse sections. Note the reduction of GATA5 in *Tie2-cre;Gata5*$^{Flox/Flox}$ embryos and its absence in *Gata5* null and *Tie2-cre;Gata5*$^{Flox/Flox}$ embryos. (**K-N**) Immunostaining of E11.5 transverse sections of control and *Tie2-cre;Gata5*$^{Flox/Flox}$ embryos for NICD and Jag1. Note the reduced expression of both antibodies in the *Tie2-cre;Gata5*$^{Flox/Flox}$ embryos. Bars = 20μm.

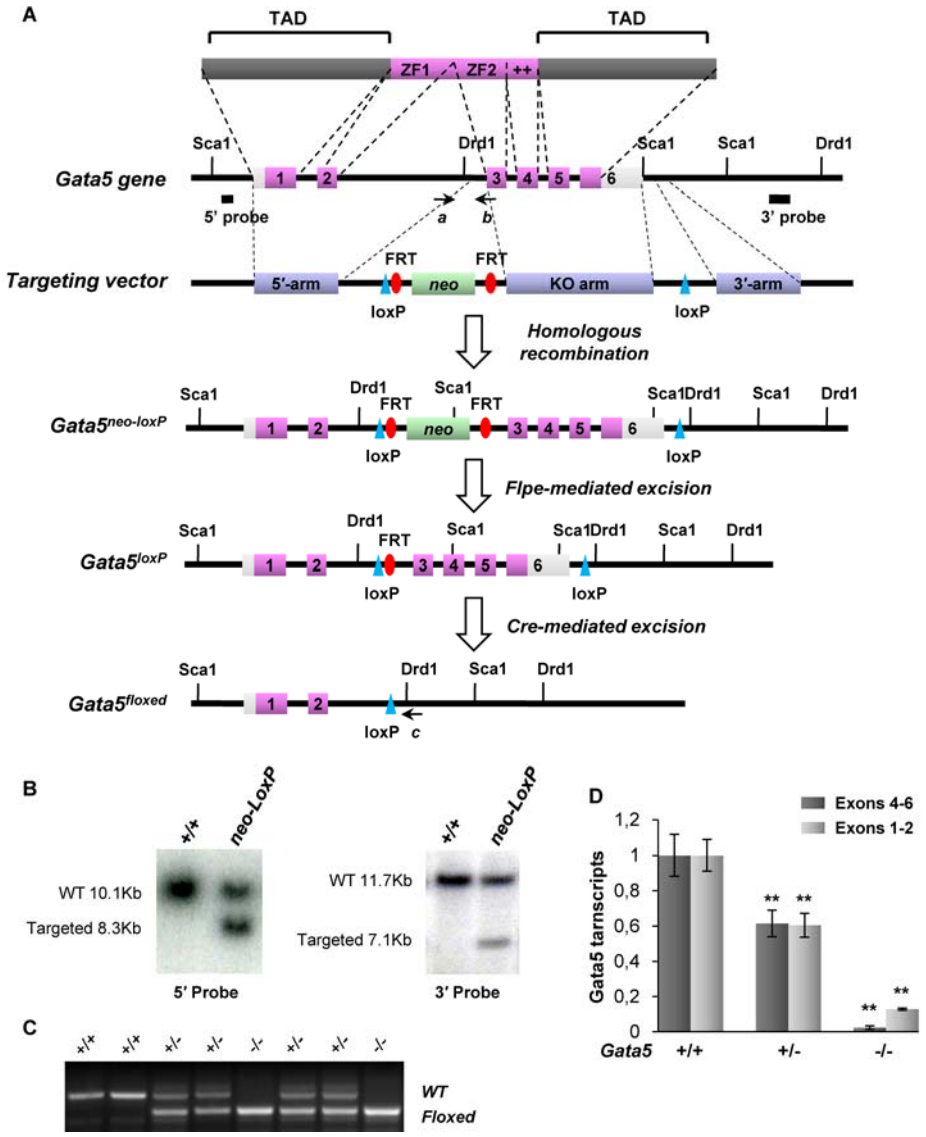


Figure 2. 1. Generation of a *Gata5* null allele

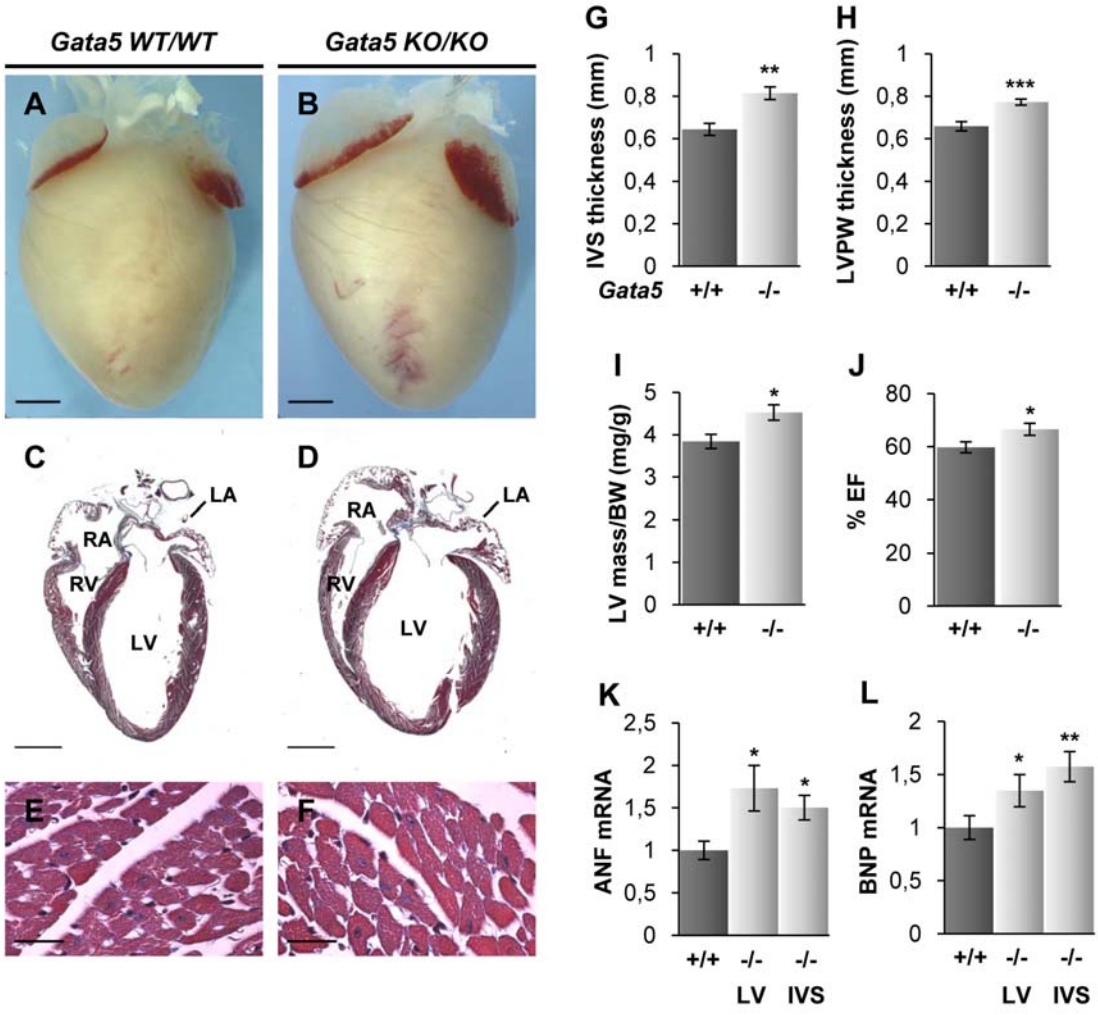
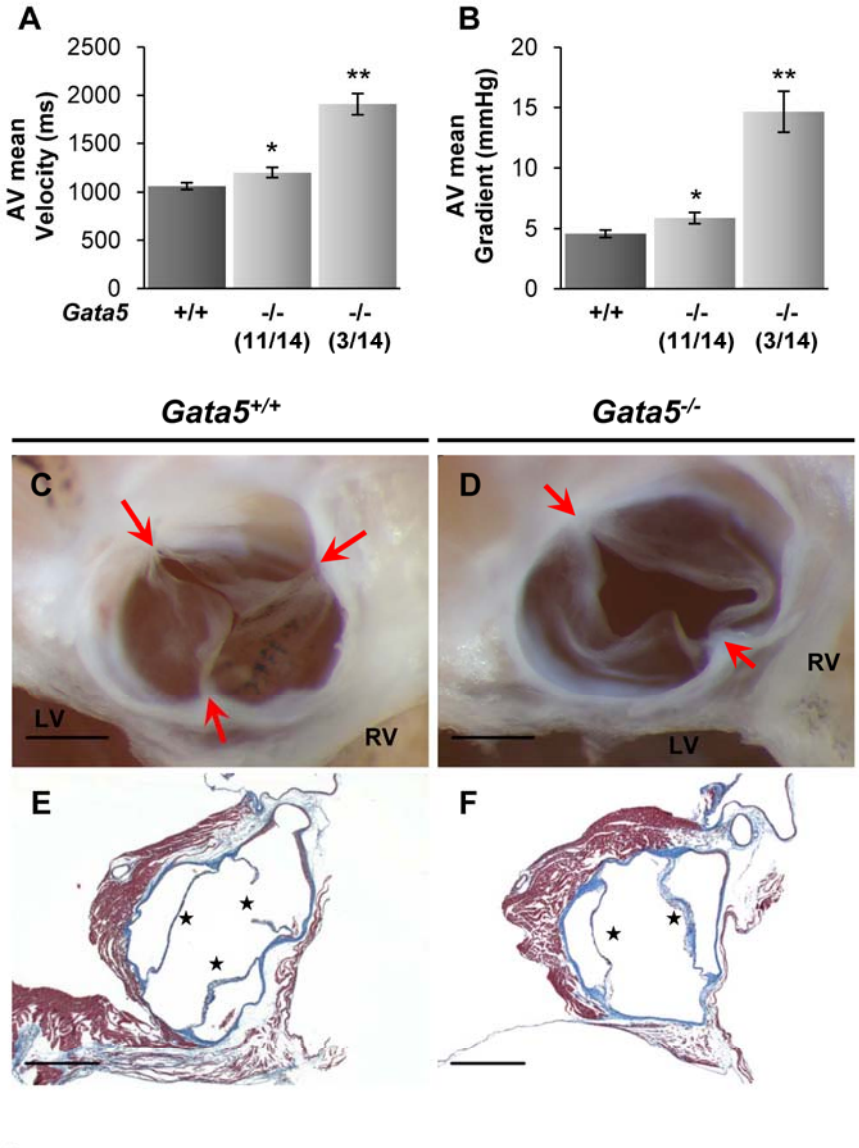


Figure 2. 2. Mild left ventricular hypertrophy of *Gata5*$^{-/-}$ mice



G

Genotype	# mice	BAV	% defect
Gata-5 +/+	29	1	3
Gata-5 -/-	28	7	25

Figure 2. 3. Valvular dysfunction of *Gata5*$^{-/-}$ mice

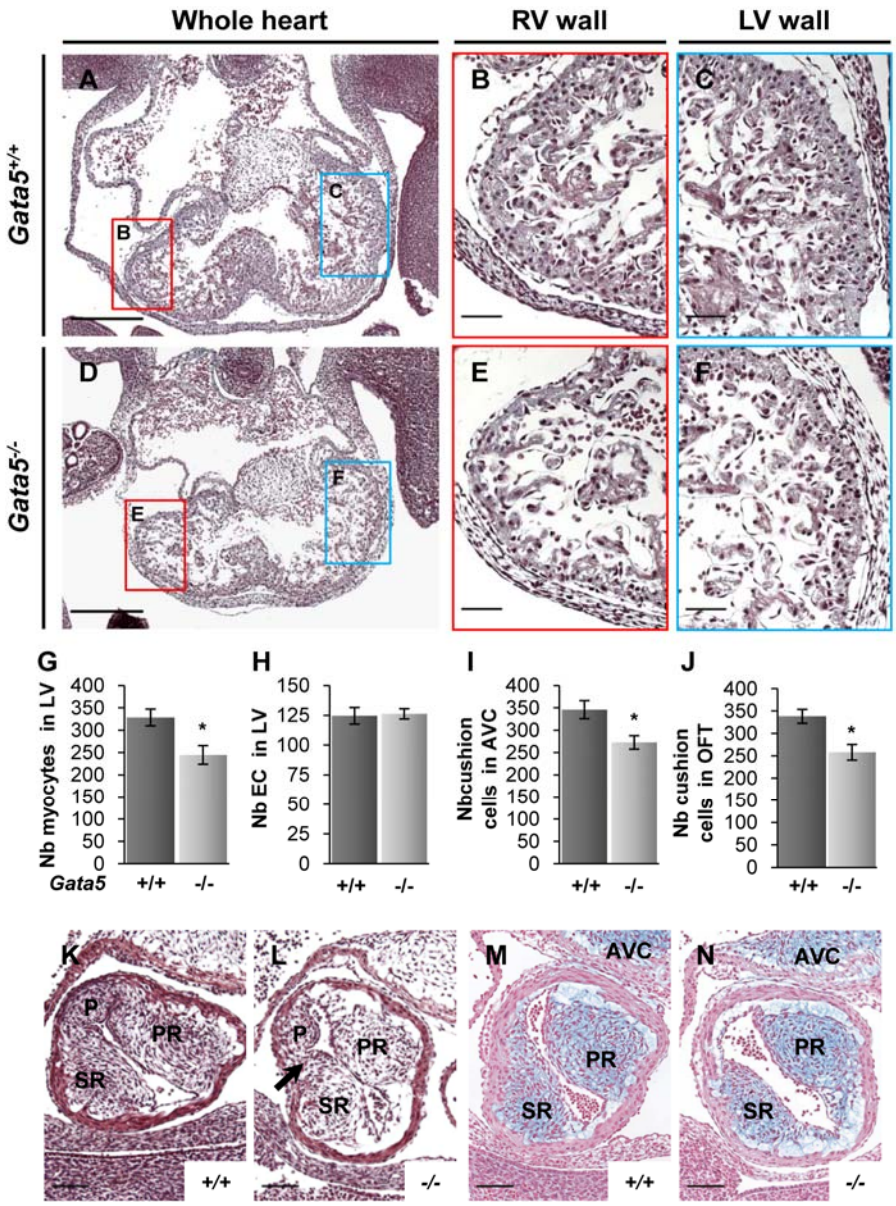


Figure 2. 4. Reduced trabeculation and R-N type of BAV in *Gata5* null embryos

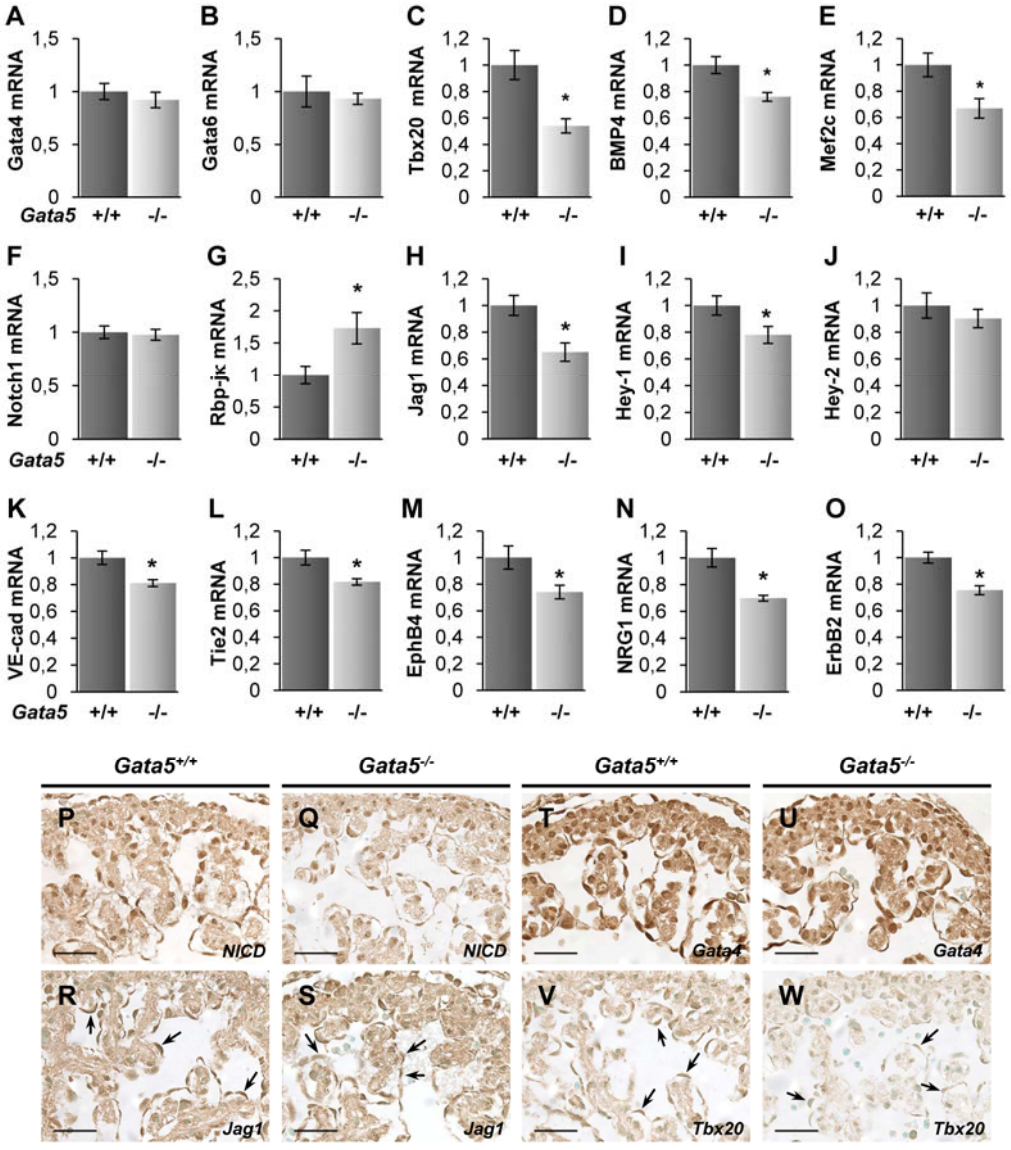


Figure 2. 5. Modulation of gene expression in *Gata5*$^{-/-}$ embryos

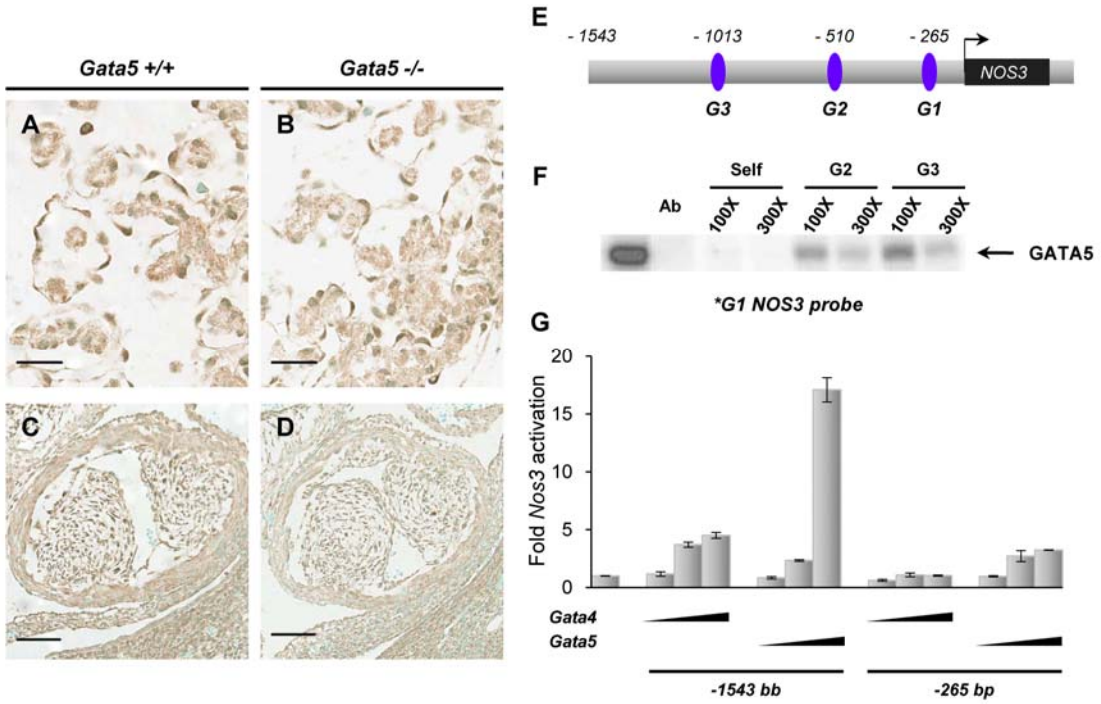


Figure 2. 6. GATA5 regulates *NOS3* expression

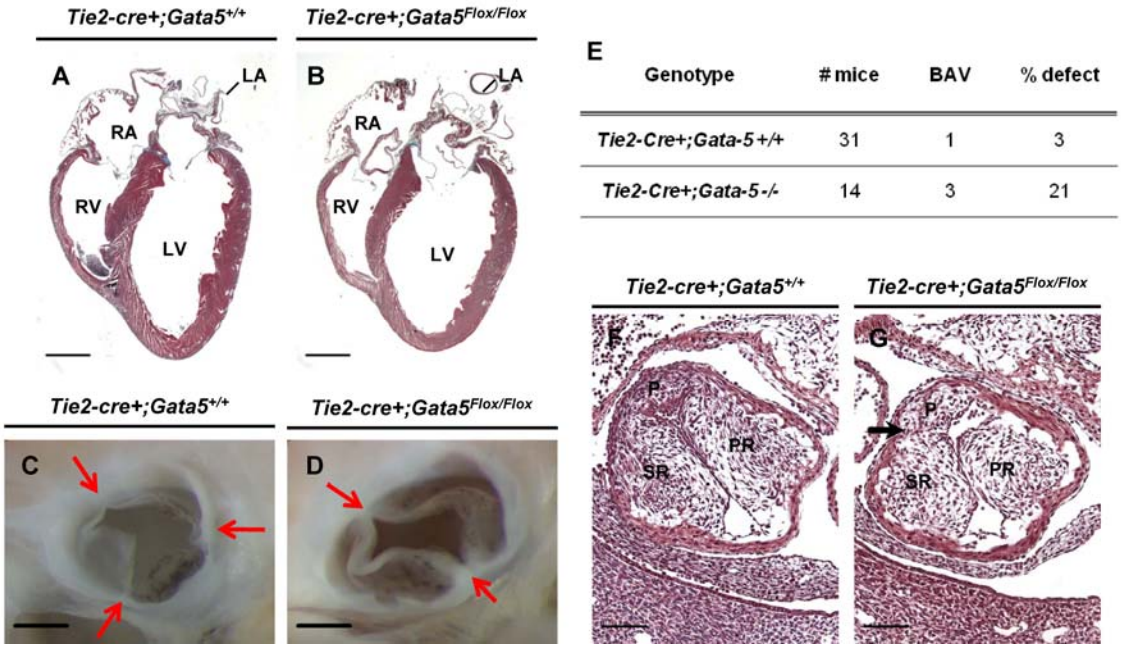


Figure 2. 7. *Gata5* is required in endocardial cells for aortic valve formation

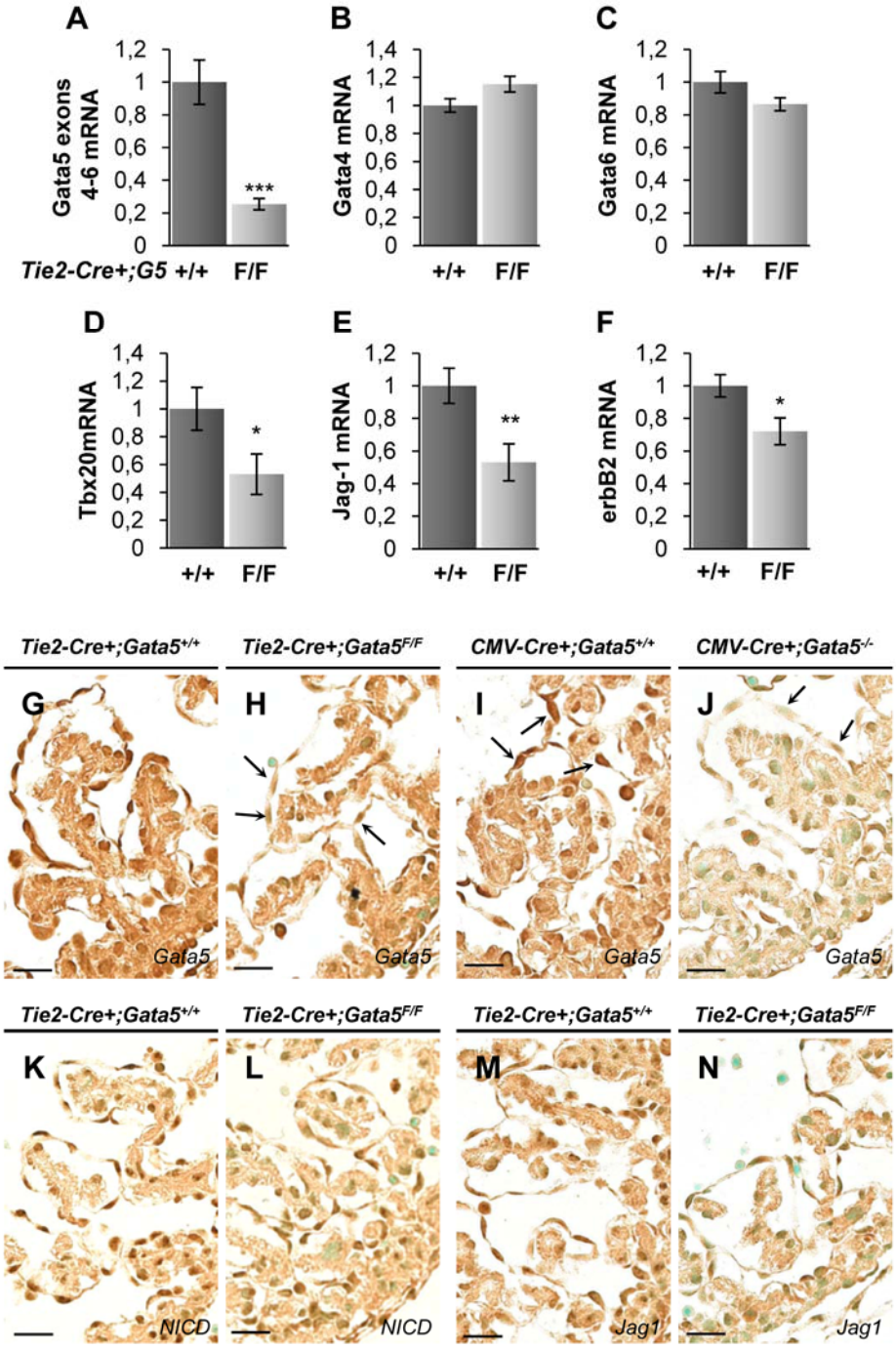


Figure 2. 8. Modulation of gene expression in *Tie2-cre;Gata5*$^{Flox/Flox}$ embryos

Chapter 3

GATA5 interacts with GATA4 and GATA6 in outflow tract development

Short title: GATA factors interaction in heart morphogenesis

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3.1. Abstract

Congenital heart disease (CHD) is the largest class of birth defects in human with a 4-5% prevalence. Human genetic studies have established that CHD is heritable with complex transmission and expressivity indicative of gene-gene and gene-environment interactions. Members of the GATA family of transcription factors are critical regulators of heart development and mutations in 2 of them, *hgata4* and *hgata6* are associated with outflow tract and septal defects in human. The heart expresses 3 GATA factors, GATA4, 5 and 6 in a partially overlapping pattern. Here, we report that whereas mice lacking a single copy of *Gata5*, *Gata4* or *Gata6* have either no detectable or subtle cardiac defects, compound *Gata4/Gata5* and *Gata5/Gata6* mutants die embryonically or perinataly due to severe CHDs. Almost all *Gata4*$^{+/-}$*Gata5*$^{+/-}$ mutant embryos have double outlet right ventricles (DORV), large ventricular septal defects (VSD) as well as hypertrophied mitral and tricuspid valves. Only 25% of double compound *Gata4/Gata5* heterozygotes survive to adulthood and these mice have aortic stenosis. Compound loss of a *Gata5* and a *Gata6* allele also leads to DORVs associated with subaortic VSDs. Expression of several transcription factors important for endocardial and myocardial cell differentiation, such as Tbx20, Nkx2.5 and Hand2, was reduced in compound heterozygote embryos. These findings suggest the existence of important genetic interactions between GATA5 and the 2 other cardiac GATA factors in endocardial cushion formation and outflow tract morphogenesis. The data identify GATA5 as a potential genetic modifier of CHD and provide insight for elucidating the genetic basis of an important class of human birth defects.

Keywords: congenital heart disease, transcription factors, heart development, double outlet right ventricle, endocardial cushion, septal defects

3.2. Introduction

Congenital heart disease (CHD) is estimated to occur at a prevalence of 4-5 % of live births and is the leading cause of death in the first year of life (Pierpont et al., 2007). Abnormal development of the outflow tract (OFT) accounts for about 12-14% of all CHDs, leading to malformations such as persistent truncus arteriosus (PTA), Tetralogy of Fallot (TOF), double outlet right ventricle (DORV) and transposition of the great arteries (TGA) (Hoffman *et al.*, 2002) Significant efforts have been deployed over the last decade to elucidate the cell and molecular mechanisms involved in CHD. Several lines of evidence suggest that CHD is heritable (Insley, 1987), but to date, only a few human genes have been linked to CHDs. Many of those are developmental regulatory genes. For example, mutations in *Nkx2.5* are associated with cases of TOF and atrial septal defects (ASD) while mutations in the *Tbx5* gene cause the Holt-Oram syndrome, an autosomal dominant disease with varying cardiac defects (Basson et al., 1997;Goldmuntz et al., 2001;Li et al., 1997;Schott et al., 1998). Moreover, mutations in *Gata4* have been associated with atrial and/or ventricular septal defects, TOF and PTA. (Garg et al., 2003;Nemer et al., 2006;Rajagopal et al., 2007). Interestingly, heterozygous mutations of *Gata4, Nkx2.5 and Tbx5* in mice recapitulate the human phenotype (Biben et al., 2000;Bruneau et al., 2001;Rajagopal et al., 2007;Winston et al., 2010). Recently, mutations in the human *Gata6* gene have been associated with PTA and TOF (Kodo et al., 2009;Lin et al., 2010b;Maitra et al., 2010). What has emerged from combined human and mouse genetic studies is that mutations in different genes can lead to similar cardiac defects while mutations in the same gene can lead to varying defects. So far it has not been possible to establish genotype-phenotype correlation, and the exact mechanisms by which specific mutations lead to CHD remain to be defined, although it is widely assumed that haploinsufficiency is often the underlying cause of disease. Despite mechanistic uncertainty, these observations are consistent with a role for the various transcription factors at various stages of cardiac development and their cooperative interactions in regulating heart formation.

The complexity of CHD is evident at both genetic and cellular levels, as multiple lineages contribute to proper heart development. The cardiogenic fields initially give rise to a linear heart tube that undergoes rightward looping to produce a four chambered heart. The first heart field contributes to the formation of the left ventricle,

the atrioventricular canal and both atrial chambers (Buckingham et al., 2005). The secondary heart field (SHF), a progenitor cell population of splanchnic and pharyngeal mesoderm that lies medial to the cardiac crescent, contribute to the formation of the right ventricle and the OFT (Kelly et al., 2001;Mjaatvedt et al., 2001;Waldo et al., 2001). Remodeling of the OFT into the distinct vessels of the aorta and pulmonary trunk requires complex interactions between the myocardium, the endocardium and cardiac neural crest cells (NCC). In response to diverse signals from the myocardium, a subset of endocardial cells in the proximal OFT will proliferate and undergo an epithelial-to-mesenchymal transformation (EMT) to invade and migrate into the cushion jelly where they will activate the mesenchymal program. Cardiac neural crest cells, originating in the dorsal part of the neural tube, will migrate ventrally through the pharyngeal arches into the distal OFT cushions, where they are required for cushion formation, septation and proper alignment of the OFT (Hutson *et al.*, 2007). Consequently, impaired development of the OFT results in conotruncal defects.

Members of the GATA family of transcription factors play important roles in differentiation, proliferation and survival of different cell types. In the heart, 3 GATA factors are present in a partially overlapping pattern (Molkentin, 2000;Nemer *et al.*, 2003;Patient *et al.*, 2002). GATA4 is highly expressed in myocytes, endocardial cells and epicardial cells of the heart. Embryos lacking *Gata4* die by E8.5 because of defects in ventral migration causing cardia bifida (Kuo et al., 1997;Molkentin et al., 1997). Analysis of rescued *Gata4*$^{-/-}$ embryos revealed cardiac defects including disrupted heart looping, absence of endocardial cushion formation, lack of a proepicardial organ and hypoplastic ventricular myocardium (Watt et al., 2004). Several studies were preformed to further investigate the role of GATA4 in endocardial or myocardial cell development. Inactivation of *Gata4* in endothelial cells causes embryonic lethality by E12.5 due to failure to promote endocardial cushion formation and remodeling (Rivera-Feliciano et al., 2006). Early myocardial specific deletion of *Gata4* results in myocardial thinning and hypoplastic endocardial cushions (Zeisberg et al., 2005). Moreover, haploinsufficiency of *Gata4* has been associated with cardiac defects including common atrioventricular canal, DORV and hypoplastic ventricular myocardium (Pu et al., 2004). GATA6 is expressed in myocytes but also in neural crest as well as endocardial and vascular smooth muscle cells (VSMC) (Nemer *et al.*, 2003). Inactivation of *Gata6* specifically in neural crest is sufficient to cause PTAs and lethality

by E18.5-P2, revealing a role for GATA6 in the patterning of the OFT and aortic arch (Lepore et al., 2005). Myocardial specific loss of both *Gata4* and *Gata6* in mice leads to acardia, suggesting that genetic interactions between these factors is essential for the onset and/or maintenance of cardiogenesis (Zhao et al., 2008). Interestingly, mice with compound heterozygous mutation in gata4 and gata6 die embryonically around e13.5 due to vascular defects; in addition, these mice display persistent truncus arteriosus, evidence of septation failure of the outflow tract as well as myocardial thinning. These results are indicative of interaction between GATA4 and 6 in cardiac and vascular development (Xin et al., 2006). In contrast to GATA4 and -6, GATA5 expression is more restricted to endocardial cells and endocardial cushions of the OFT and atrioventricular canal during heart development. The dynamic expression of GATA5 in endocardial cells suggests a specific function for this transcription factor in endocardial development. Consistent with this, *faust* (which encode GATA5) mutants in zebrafish have *cardia bifida* and lack endocardial cells (Reiter et al., 1999). In addition, downregulation of *Gata5* in an *in vitro* model of endocardial differentiation inhibits terminal differentiation and expression of endocardial differentiation markers (Nemer *et al.*, 2002). An important role for GATA-5 in endocardial differentiation is further supported by recent findings showing that lack of *Gata 5* in mice leads to bicuspid aortic valve formation (Laforest et al., 2010). Thus, all three cardiac GATA factors appear to play important functions in endocardial cushion development and/or outflow tract morphogenesis.

Because expression of the three cardiac GATA factors partially overlaps and since they can bind similar DNA regulatory elements and activate common target promoters through heterotypic interactions (Charron *et al.*, 1999;Nemer *et al.*, 2003), we tested whether GATA5 might genetically interact with GATA4 and GATA6 in OFT development. Here we show that whereas mice lacking a single copy of *Gata4*, *Gata5* or *Gata6* have subtle or no cardiac malformations, Gata4$^{+/-}$*Gata5*$^{+/-}$ and *Gata5*$^{+/-}$*Gata6*$^{+/-}$ double heterozygote mice die embryonically or perinataly due in large part to profound defects of OFT development. The *Gata4/Gata5* compound heterozygotes had an array of cardiac defects including DORVs, large membranous VSDs, hypertrophied atrioventricular valves and complete atrioventricular canal defects. On the other hand, the *Gata5*$^{+/-}$*Gata6*$^{+/-}$ double heterozygote embryos had DORVs associated with subaortic VSDs. Gene expression analysis revealed altered mRNA levels of several cardiac regulators and markers of differentiation including *Nppb*, *Myh6*, *Nkx2.5* and

Tbx20. These findings reveal the existence of important genetic interactions between GATA5 and the other cardiac GATA factors in the formation of the septum intermedium which contribute to the membranous ventricular septum and to the tricuspid and mitral valves; they also underscore the critical role of GATA4,-5 and -6 OFT development. The results raise the possibility that subtle alterations in the level or activity of any 2 cardiac GATA factors might lead to human CHD.

3.3. Materials and methods

Animals

Mice handling and experimentation were performed in accordance with institutional guidelines. All protocols were approved by the institutional animal care committees.

Mice heterozygous for *Gata4*, *Gata5* and *Gata6* were generated and genotyped as previously described (Aries et al., 2004;Koutsourakis et al., 1999;Laforest et al., 2010). All lines were maintained in the C57/BL6 background. To generate double heterozygotes, mice heterozygous for *Gata4* or *Gata6* were mated to *Gata5* heterozygote mice and pregnant mothers or newborn litters were sacrificed at various embryonic timepoints and postnatal timepoints. The morning a vaginal plug was observed was defined as embryonic day (E) 0.5.

For histology, whole embryos were fixed in 4% paraformaldehyde at 4°C overnight, dehydrated through graded ethanol series, embedded in paraffin and sectioned at 4-µm intervals. Masson's trichrome staining was carried out on heart sections using standard procedures to visualize defects.

Quantitative polymerase chain reaction (Q-PCR)

Total RNA was isolated from whole hearts at E12.5 with TRIZOL reagent (Invitrogen) according to the manufacturer's instruction. cDNAs were generated from 2 ug of total RNA using the Omniscript RT kit (Qiagen). QPCR was performed with cDNA diluted 1/100 using the Quiagen QPCR kit. Briefly, DNA template and 400 nM oligonucleotides were used at an annealing temperature of 58°C using the Quantitect SYBR green PCR kit (QIAGEN) in an MX3500 real-time PCR machine (Stratagene, La Jolla, CA). Mean gene expression was corrected by GAPDH and calculated from wildtype and double heterozygotes embryos (n = 4-7 per group). Primers sequence is available on request.

Statistical analysis

Values are presented as mean ±S.E.M. and n refers to the number of mice per group. P values were generated using the Student's two-tailed t-test, and statistical significance was considered as $P<0.05$.

3.4. Results

Reduced viability of Gata5$^{+/-}$Gata4$^{+/-}$ embryos

Both GATA4 and GATA5 are expressed in the endocardial cushions at the same embryonic stages (between E9-E12). We investigated possible *in vivo* interactions between them in heart development by crossing mice heterozygous for either a *Gata5* or a *Gata4* allele. According to Mendelian transmission, equal ratios of wildtypes, *Gata4* heterozygotes, *Gata5* heterozygotes and *Gata4/Gata5* double heterozygotes were expected. However, at weaning, *Gata4$^{+/-}$Gata5$^{+/-}$* double heterozygotes (G4/G5 hets) were obtained at far lower frequency than expected (6% vs 25%) (Figure 1A). Analysis of embryos from timed matings suggested high perinatal lethality of G4/G5 hets, although decreased viability was evident starting at E14.5 (Figure 1A). Moreover, growth retardation was observed in all *Gata4$^{+/-}$Gata5$^{+/-}$* embryos as early as E11.5 eventhough mice with this genotype were present at the expected Mendelian ratios (Figure 1B-F). By E15.5, growth retardation was more evident and reduced vascularization was often observed in *Gata4$^{+/-}$Gata5$^{+/-}$* heterozygotes (Figure 1G-K). Moreover, 25% of *Gata4$^{+/-}$Gata5$^{+/-}$* embryos were dead at E15.5 (Figure 1K).

Gata4$^{+/-}$Gata5$^{+/-}$ double heterozygotes display DORVs and VSDs

In order to determine the cause of lethality, histological analyses were performed on E15.5 *Gata4$^{+/-}$Gata5$^{+/-}$* double heterozygotes. They revealed profound structural cardiac defects. A double outlet right ventricle (i.e. the aortic valve opens in the right ventricle instead of the left ventricle) as well as large ventricular septal defects were evident in 86% of embryos (Figure 2D, H, L). This was not observed in wildtype or *Gata5$^{+/-}$* heterozygote littermates and only 29% of *Gata4$^{+/-}$* had a DORV (Table 1). Moreover, some *Gata4$^{+/-}$Gata5$^{+/-}$* embryos (2/7 embryos) had a complete atrioventricular canal defect, meaning a single atrioventricular valve associated with atrial and ventricular septal defects (Table 1). Wildtype and *Gata5$^{+/-}$* heterozygote littermates did not demonstrate this defect and had two separate atrioventricular valves while 1/7 *Gata4$^{+/-}$* embryo (only 14%) had a common atrioventricular valve. As both GATA4 and GATA5 are expressed in the atrioventricular (AV) cushion, we were interested to know if the valve morphology was normal. We found that the tricuspid and mitral valves of *Gata4$^{+/-}$Gata5$^{+/-}$* double heterozygotes were hypertrophied compared to control littermates, a situation that could cause stenosis (Table 1). As these defects are

all thought to arise from abnormal development of endocardial cushions, the results suggest that GATA4 and GATA5 genetically interact for proper EC development.

G4/G5 heterozygotes display myocardial thinning and smaller AV endocardial cushions

Growth retardation was already visible in *Gata4*$^{+/-}$*Gata5*$^{+/-}$ heterozygotes at E11.5. Histological analysis of tissue sections at this stage revealed smaller hypotrabeculated hearts in *Gata4*$^{+/-}$*Gata5*$^{+/-}$ compared to single heterozygous or wildtype littermates (Figure 3A-D). Higher magnification of the left ventricle also revealed a reduction in the ventricular compact zone thickness (Figure 3E-H). Cell counting indicated significant decreases in the number of myocytes and endocardial cells in the left ventricle of G4/G5 hets at E11.5 (Figure 3M). By E15.5, 71% (5/7) *Gata4*$^{+/-}$*Gata5*$^{+/-}$ embryos had myocardial thinning of the left and right ventricles (data not shown). However, because similar ventricular hypoplasia was also observed in the *Gata4*$^{+/-}$ embryos at E11.5 and E15.5, GATA5 does not appear to worsen the myocardial defects of GATA4 haploinsufficient hearts (Figure 3F).

At E15.5 *Gata4*$^{+/-}$*Gata5*$^{+/-}$ embryos show AV canal defects, VSDs and hypertrophied AV valves. These structures arise from endocardial cushion and GATA4 and GATA5 are co-expressed in the AV and OFT cushions between E9.5 and E12.5. Consistent with a role for both factors therein, endocardial cushions of *Gata4/Gata5* compound embryos were smaller but properly formed (Figure 3I-L). *Gata4*$^{+/-}$*Gata5*$^{+/-}$ mutant embryos showed a larger reduction in the total number of cushion cells within the atrioventricular endocardial cushion compared to *Gata4*$^{+/-}$, *Gata5*$^{+/-}$ and wildtype littermates. These results indicate that GATA4 and GATA5 cooperate in endocardial cushion formation in the AV canal.

QPCR analysis on RNA isolated from hearts of WT and *Gata4/Gata5* compound mutants at E12.5 revealed that *Gata4* and *Gata5* expression was reduced by 30% and 60% compared to WT littermates with no change in *Gata6* levels (Figure 4). Interestingly, we noticed an upregulation of *BNP* transcripts, possibly indicative of early cardiac stress. The level of other cardiac genes was also altered. *Myh6* but not *Myh7* transcripts were downregulated in the hearts of compound mutants compared to wildtype controls. *Mef2c and Nkx2.5* transcripts were also significantly decreased. Moreover, we observed decreased mRNA expression of *Tbx20* and *erbB2*, two genes

involved in endocardial cushion formation. No significant alteration in the level of other genes implicated in endocardial cushion formation like *Tgfβ1*, *Tgfβ2*, *Bmp2*, *Bmp4*, *erbB3*, *Notch1*, *Jag-1*, *Hey-1* and *Hey-2* was evident (data not shown).

Adult Gata4/Gata5 compound mutants have aortic stenosis

Only 25% of *Gata4*$^{+/-}$*Gata5*$^{+/-}$ double heterozygotes survive to adulthood; we were interested in assessing cardiac structure and function in this subgroup. Echocardiographic measurements on 70 days old mice revealed increased left ventricular mass and significantly decreased ejection fraction in adult G4/G5 hets compared to wildtype littermates (Figure 5A, B). Hemodynamic evaluation revealed increased mean pressure gradient through the aortic valve of *Gata4*$^{+/-}$*Gata5*$^{+/-}$ double heterozygotes (Figure 5C). As shown on the graph, 66% (2/3) of G4/G5 hets mice had a very high pressure gradient compared to wildtype, *Gata4*$^{+/-}$ and *Gata5*$^{+/-}$ littermates. These observations suggested the presence of aortic valve constriction. Histological analysis of these hearts confirmed the presence of left ventricular hypertrophy compared to wildtype and *Gata4*$^{+/-}$ and *Gata5*$^{+/-}$ littermates and revealed massively hypertrophied aortic valve in adult *Gata4*$^{+/-}$*Gata5*$^{+/-}$ double heterozygotes (Figure 5G). Together, the results are consistent with defective valve development leading to aortic stenosis.

Disrupted valvulogenesis in Gata5*$^{+/-}$*Gata6*$^{+/-}$ *embryos

GATA5 also overlaps with GATA6 in outflow tract cushions. To determine if GATA6 and GATA5 genetically interact, we generated *Gata5*$^{+/-}$*Gata6*$^{+/-}$ mice. Only 1% of the expected *Gata5*$^{+/-}$*Gata6*$^{+/-}$ double heterozygotes (G5/G6 hets) were present at weaning (postnatal day 30), suggesting that the combined mutations result in embryonic or perinatal lethality (Figure 6A). Analysis of 13 post-natal litters revealed that 47 % (10/21 embryos) of mice heterozygous for both *Gata5* and *Gata6* were dead at birth. Analysis of E11.5, E15.5 and E18.5 embryos indicated reduced Mendelian ratios and slight growth retardation starting at E14.5-E15.5 (Figure 6A, F-J).

To determine the etiology of the embryonic and perinatal lethality of *Gata5*$^{+/-}$ *Gata6*$^{+/-}$ embryos, we sectioned embryos at E15.5, when both ventricles are completely septated and the four valves of the heart have formed. Histological analysis at this stage demonstrated the presence of a DORV in 86% of *Gata5*$^{+/-}$*Gata6*$^{+/-}$ embryos (Figure 7D, H). Similarly to the Gata4/Gata5 compound embryos, we also found the

presence of VSDs but only at the level of the aortic valve (Figure 7L). The VSD was present over 30-50 μm. We also noticed that a subset of *Gata5*$^{+/-}$*Gata6*$^{+/-}$ embryos had increased thickness of the left ventricular compact zone compared to WT, *Gata5*$^{+/-}$ and *Gata6*$^{+/-}$ littermates (Figure 7M). The AV and semilunar valves were formed properly and did not show signs of hypertrophy. No other cardiac defects were noted. A summary of the phenotypes is given in table 2. Together, the data reveal important genetic interactions between GATA5 and GATA6 in outflow tract development.

Gene expression studies were performed on RNA isolated from hearts of wildtype and *Gata5*$^{+/-}$*Gata6*$^{+/-}$ mutants at E12.5. Expression of *GATA5* and *GATA6* transcripts was reduced by 40-50% in compound mutants while the expression of *GATA4* was not significantly altered (Figure 8). In contrast to what was observed in the G4/G5 hets, *BNP* mRNA was downregulated. Similarly, a strong reduction in *Myh6* but not *Myh7* transcript abundance was observed in double het embryos. A 50% reduction in Nkx2.5 and Tbx20 mRNA expression, identical to that found in the *Gata4*$^{+/-}$*Gata5*$^{+/-}$ embryos was noted. Lastly, we observed a 2-fold increase in *Raldh2* mRNA expression, which could potentially explain the presence of DORV as excess retinoic acid signalling leads to conotruncal malformations such as TGA, DORV and TOF (Nakajima et al., 1996). A significant 30% decrease of *Hand2* transcripts was also observed in *Gata5/Gata6* compound embryos.

3.5. Discussion

Transcription factors GATA4, GATA5 and GATA6 are expressed at various stages of heart development and are important cardiac regulators(Nemer *et al.*, 2010). In the present study, we show that compound haploinsufficiency of *Gata5* and either *Gata4* or *Gata6* is incompatible with embryonic development and perinatal survival due to defective heart formation. The cardiac phenotype of *Gata4*$^{+/-}$*Gata5*$^{+/-}$ heterozygotes include DORV, very large VSDs, complete AV canal defects and mitral and tricuspid stenosis. The *Gata4/Gata5* compound heterozygotes who survive to adulthood show signs of aortic stenosis. Similarly, compound haploinsufficiency of *Gata5* and *Gata6* leads to cardiac anomalies that include DORVs and VSDs. The defects observed in the double heterozygote embryos are distinct from those found in their single heterozygote parents, indicative of a cooperative function for GATA factors during cardiac morphogenesis. The phenotype of the *Gata4*$^{+/-}$*Gata5*$^{+/-}$ heterozygotes reported here differs from the one recently published by Singh K *et al* (Singh et al., 2010). In their study, Singh K *et al* showed that *Gata4*$^{+/-}$*Gata5*$^{+/-}$ embryos had myocardial thinning by E14.5 but were viable. It was only when they generated *Gata4*$^{+/-}$*Gata5*$^{-/-}$ embryos that they obtained a more severe phenotype that included AV canal defects and myocardial thinning similar to our G4/G5 hets. This indicates increased severity of the phenotype in our crosses, consistent with the heightened phenotype of our *Gata5* null mice as compared to theirs (Laforest et al., 2010). Previously, haploinsufficiency of both GATA4 and GATA6 revealed an important role for these 2 GATA factors in cardiomyocyte proliferation and OFT septation (Xin et al., 2006;Zhao et al., 2008). While we had no evidence for genetic interaction between GATA5 and either of the other 2 cardiac GATA proteins in myocardial proliferation there was clear evidence of genetic interaction in cushion formation and OFT septation. Together with these studies, our present work underscores the exquisite sensitivity of the OFT to GATA protein dosage and reveals the importance of cooperative interactions between any 2 cardiac GATA factors in regulating various events of cardiovascular development.

Cooperative roles of GATA4, 5 and 6 in cardiac development

The majority of *Gata4/Gata5* and *Gata5/Gata6* double heterozygote embryos developed DORVs, which is thought to occur from abnormal rotation of the OFT. Results of previous work have suggested that the myocardium of the OFT and the right

ventricle are derived from a population of cells located within the secondary heart field (Kelly et al., 2001;Mjaatvedt et al., 2001;Waldo et al., 2001). According to Bajolle F *et al*, the myocardium at the base of the OFT is required for normal positioning of the great vessels (Bajolle et al., 2008). Germline *Gata4* and *Gata6* mutant mice die too early embryonically to be able to evaluate their function in OFT morphogenesis (Koutsourakis et al., 1999;Kuo et al., 1997;Molkentin et al., 1997). *Gata4* hypomorphs have DORVs, clearly indicating that GATA4 is required in rotation of the OFT embryonically (Crispino et al., 2001;Pu et al., 2004). However, this phenotype was not produced when *Gata4* was disrupted in the SHF leading the authors to suggest that GATA5 and GATA6 might compensate for GATA4 in the SHF as they were robustly expressed in the pharyngeal mesoderm (Rojas et al., 2008). Similarly, deletion of *Gata6* in cardiac crest cells using *SM22*-Cre or *Wnt1*-Cre reporter mice resulted in the formation of PTAs and DORVs. Moreover, *Gata4/Gata6* compound heterozygosity results in the formation of a single OFT vessel. These observations, coupled with our results, clearly indicate that OFT defects seem to be a common consequence of loss of two GATA alleles, suggesting that a threshold of GATA factors is essential in the rotation and patterning of the OFT. Whether or not this reflects action of GATA factors in the same cell type (SHF or endocardial derived) or complementation of a defective pathway arising from 2 distinct lineages remain to be defined.

Endocardial cushion defects of Gata5$^{+/-}$Gata4$^{+/-}$ embryos

Our work demonstrates that concomitant loss of one allele of *Gata4* with one allele of *Gata5* leads to VSDs, AV canal defects and AV valve hypertrophy with variable penetrance. These anomalies are thought to arise from abnormal endocardial cushion formation and are consistent with the presence and role of GATA4 and GATA5 in endocardial cell expansion and differentiation (Nemer *et al.*, 2002;Rivera-Feliciano *et al.*, 2006). The process of endocardial cushion formation starts at E9.5 in the mouse and requires complex interactions between the endocardium and myocardium. In response to diverse signals, endocardial cells go through an epithelial-to-mesenchymal transformation (EMT) and migrate into the cushion jelly; later in development these cushions will be remodeled to form the interventricular septum, atrial septum and the valves of the hearts (Person et al., 2005). In the compound *Gata4/Gata5* heterozygotes, AV cushion formation appears relatively normal at E11.5. However, we noticed reduced number of cushion cells within the endocardial cushion, similar to the

decreased number of mesenchymal cells in the AV cushion of *Gata5* null mice, suggestive of a role for GATA5 in cushion formation (Laforest et al., 2010). By E15.5, a subset of *Gata5*$^{+/-}$*Gata4*$^{+/-}$ embryos had AV canal defects, indicating that later remodelling of the cushion did not occur. Complete AV canal defects were found in only one *Gata4*$^{+/-}$ embryo but the penetrance was higher in the double heterozygotes, likely indicating that GATA4 and GATA5 interact together in cushion formation. Whether these interactions involve myocardial-endocardial crosstalk or reflect cell autonomous function in the endocardium where both factors are expressed will need to be clarified. Generation of *Gata4/Gata5* double heterozygotes in which GATA4 and GATA5 are deleted only in endocardial cells will help address this question.

GATA downstream targets

The cardiac GATA factors have been shown to regulate a large number of cardiac genes including *ANF*, *BNP*, *Mef2c*, *Nkx2.5*, *Bmp4*, *Myh6* and *Myh7* (Charron *et al.*, 1999;Dodou *et al.*, 2004;Grepin *et al.*, 1994;Nemer *et al.*, 2003). Moreover, GATA4 and GATA6 are able to heterodimerize and synergistically activate several of these promoters (Charron et al., 1999). *Myh6* null mice die between E11.0-E12.0 due to cardiac defects including hypoplasia of the right ventricle coupled with hypotrabeculation, indicating that Myh6 plays an important role in vertebrate heart development (Jones et al., 1996). Here, we show that *Myh6* gene expression is dramatically downregulated in the hearts of compound *Gata4/Gata5* and *Gata5/Gata6* heterozygotes. In addition, haploinsufficiency of *Gata5* combined with loss of either a *Gata4* or *Gata6* allele resulted in reduced expression of *Nkx2.5*, *Tbx20*, *Mef2c*, *Hand2* and *erbB2*, indicating that these genes may be more sensitive to combined loss of 2 GATA factors. Loss of Hand2 results in embryonic lethality and absence of right ventricle formation (Srivastava et al., 1997). Hand2 is expressed in myocardial, endocardial and neural crest lineages. Interestingly, deletion of Hand2 from neural crest cells is sufficient to produce an identical phenotype as the one reported here (i.e. DORVs and VSDs) possibly through regulation of proliferation and differentiation of SHF derived cells (Holler *et al.*, 2010;Morikawa *et al.*, 2008). Moreover, Hand2 transcription was shown to be GATA dependent (McFadden et al., 2000). Similarly, loss of Tbx20 revealed a critical role in OFT development (Takeuchi et al., 2005). Tbx20 is expressed in both myocardial and endocardial cells where it is required for proliferation and differentiation (Shelton *et al.*, 2008). A role for GATA factors in

regulating Tbx20 has not yet been reported in the mammalian heart but in Drosophila, the Tbx20 ortholog, neuromancer was shown to be a downstream mediator of the GATA factor pannier in the heart (Qian *et al.*, 2009). On the other hand, Nkx2.5 transcription is GATA dependent and loss of Nkx2.5 causes septal, valvular and myocardial dysmorphogenesis (Biben et al., 2000;Grepin et al., 1997;Lien et al., 1999). Interestingly, mutations in Nkx2.5 are among the most common monogenic loci associated with DORV in human (Obler et al., 2008). Lastly, excess retinoic acid signaling specifically inhibit OFT cushion remodeling while *Raldh2* null mice have cardiac abnormalities due to defective SHF cell differentiation (Lin et al., 2010a;Nakajima et al., 1996). Thus, dysregulation of these genes could explain several of the cardiac phenotypes observed in the double GATA heterozygote embryos.

GATA4, GATA5 and GATA6 in human CHD

Heterozygous mutations in *Gata4* and *Gata6* have been linked to congenital heart defects in humans (Garg et al., 2003;Kodo et al., 2009;Nemer et al., 2006;Pehlivan et al., 1999;Zhang et al., 2008). The CHDs associated with mutations in *Gata4* are predominantly ASDs, VSDs but DORV, TOF and pulmonary stenosis have also been reported. Recently, mutations in *Gata6* in humans have been associated with PTA. To date, no GATA5 mutations have been reported in human CHD.

Despite the significant efforts of the past decade to elucidate the molecular mechanisms and the genetic basis of congenital heart defects, the fundamental causes for the majority of CHD remain unidentified. CHD has an increased risk of recurrence within families; among affected relatives, the cardiac phenotypes are usually different and occur with variable penetrance and expressivity, likely indicating that modifying factors including genetic and environmental influence the phenotype (Gill et al., 2003). Consistent with this, influences of the genetic background on the phenotype is now well documented in experimental animal models and humans (Bruneau et al., 2001;Rajagopal et al., 2007;Winston et al., 2010). The results of the present study suggest that *Gata5* may be an important genetic modifier that could potentially contribute to human CHD and more specifically to defects of the OFT.

3.6. Acknowledgements

The authors are grateful to Chantal Lefebvre and Janie Beauregard for assistance with management of mice colonies, to Annie Vallée and Geneviève Brindle for help with histology and to Dr Marie Kmita and members of the Nemer lab for discussions and helpful suggestions. This work was supported by grants from the Canadian Institutes of health research.

3.7. Figure legends

Figure 3.1. Reduced viability of *Gata4*$^{+/-}$*Gata5*$^{+/-}$ heterozygotes. (*A*) Frequency of genotypes obtained from intercrossing *Gata4*$^{+/-}$ and *Gata5*$^{+/-}$ mice. Note the reduced viability of compound *Gata4*$^{+/-}$*Gata5*$^{+/-}$ heterozygotes at weaning and the reduced frequency of genotypes (by 25%) in *Gata4*$^{+/-}$*Gata5*$^{+/-}$ starting at E14.5; another 50% is lost perinatally. (*B-K*) Growth retardation starting at E11.5 (*E, F*) and aberrant vascularization at E15.5 (*J, K*) in *Gata4*$^{+/-}$*Gata5*$^{+/-}$ heterozygotes as compared to their littermates. Embryonic lethality of 30% of *Gata4*$^{+/-}$*Gata5*$^{+/-}$ is noted as shown in a representative embryo (*K*). Bars = 1000 μm (*B-F*) and 2000 μm (*G-K*).

Figure 3.2. Cardiac defects in *Gata4*$^{+/-}$*Gata5*$^{+/-}$ heterozygotes. Trichrome staining of transverse sections of E15.5 embryos. Note how in wildtype and single hets (*A-C*, *E-G*), the aortic valve opens in the left ventricle whereas in *Gata4*$^{+/-}$*Gata5*$^{+/-}$ embryos, the aortic valve opens in the right ventricle (*D, H*), a phenotype known as double outlet right ventricle (DORV). (*I-L*) Transverse section through the heart showing an intact ventricular septum in wildtype, *Gata4*$^{+/-}$ and *Gata5*$^{+/-}$ embryos and a membranous ventricular septal defect (VSD) in *Gata4*$^{+/-}$*Gata5*$^{+/-}$ embryos (arrow in *L*). Bars = 300μm (*A-D* and *I-L*) and 100μm (*E-H*). AV, aortic valve; LV, left ventricle; RV, right ventricle.

Figure 3.3. Ventricular wall and endocardial cushion defects in *Gata4*$^{+/-}$*Gata5*$^{+/-}$ embryos. Trichrome staining of transverse sections of wildtype, *Gata4*$^{+/-}$, *Gata5*$^{+/-}$ and *Gata4*$^{+/-}$*Gata5*$^{+/-}$ E11.5 embryos. (A-D) Note ventricular wall thinning and hypotrabeculation in double hets as well as in Gata4 het. Bars = 200 μm. (E-H) Higher magnification of the left ventricular wall. Bars = 75 μm. (I-L) Transverse section through the AV endocardial cushions of WT, *Gata4*$^{+/-}$, *Gata5*$^{+/-}$ and *Gata5*$^{+/-}$ *Gata5*$^{+/-}$ embryos at E11.5. Endocardial cushions of *Gata4*$^{+/-}$*Gata5*$^{+/-}$ embryos are properly formed although a bit smaller. Bars = 100 μm. (M) Quantification of the number of cells in the left ventricle or AV canal (* $P<0.05$, ** $P<0.001$, *** $P<0.0001$). Statistical significance is compared to WT controls. AVC, atrioventricular canal; LV, left ventricle.

Figure 3.4. Modulation of gene expression in *Gata4*$^{+/-}$*Gata5*$^{+/-}$ embryos. QPCR analysis performed on whole hearts of E12.5 embryos. Transcript levels are normalized to GAPDH in each sample. Note the downregulation of *Myh6*, *Mef2c*,

Nkx2.5, *Tbx20* and *erbB2* transcripts and the upregulation of *BNP* transcripts in *Gata4*$^{+/-}$ *Gata5*$^{+/-}$ embryos compared to wildtype littermates (* P<0.05; n = 5-6 per group).

Figure 3.5. Aortic stenosis in adult *Gata4*$^{+/-}$*Gata5*$^{+/-}$ heterozygotes. (A) Echocardiography of wildtype, *Gata4*$^{+/-}$, *Gata5*$^{+/-}$ and *Gata4*$^{+/-}$*Gata5*$^{+/-}$ mice at 70 days (* P<0.5, n = 3-6 per group). Note the increase in LV mass over body weight for *Gata4*$^{+/-}$ *Gata5*$^{+/-}$ mice, suggesting left ventricular hypertrophy. (B) Echocardiography of wild-type, *Gata4*$^{+/-}$, *Gata5*$^{+/-}$ and *Gata4*$^{+/-}$*Gata5*$^{+/-}$ mice at 70 showing increased pressure gradients through the aortic valve (AV) (n = 5 for WT, *Gata4*$^{+/-}$ and *Gata5*$^{+/-}$ littermates). Two out of three *Gata4/Gata5* compound heterozygotes have very high mean pressure gradient through the aortic valve. (C-F) Trichrome staining of a frontal section from 90 days old mice. Note the increased ventricular hypertrophy of *Gata4/Gata5* compound heterozygotes. Bar = 1000 μm. LA, left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle. (G-J) Trichrome staining of a frontal section of the aortic valve in these same mice. Note the hypertrophied aortic valve in *Gata4*$^{+/-}$*Gata5*$^{+/-}$ heterozygotes compared to wildtype, *Gata4*$^{+/-}$ and *Gata5*$^{+/-}$ littermates. Bar = 300 μm.

Figure 3.6. Reduced viability of *Gata5*$^{+/-}$*Gata6*$^{+/-}$ heterozygotes. (*A*) Frequency of genotypes obtained from intercrossing *Gata5*$^{+/-}$ and *Gata6*$^{+/-}$ mice. Embryonic and perinatal lethality of *Gata5*$^{+/-}$*Gata6*$^{+/-}$ heterozygotes is demonstrated by the reduced frequencies of *Gata5*$^{+/-}$*Gata6*$^{+/-}$ embryos at various developmental stages. (*B-J*) Dissection and visual inspection of *Gata5*$^{+/-}$*Gata6*$^{+/-}$ heterozygotes. Mild growth retardation is visible at E15.5 (*F-J*). Bars = 1000 μm (*B-E*), 2000 μm (*F-J*).

Figure 3.7. Cardiac defects of *Gata5*$^{+/-}$*Gata6*$^{+/-}$ heterozygotes. Trichrome staining of transverse sections of E15.5 wildtype (*A, E, I*), *Gata5*$^{+/-}$ (*B, F, J*), *Gata6*$^{+/-}$ (*C, G, K*) and *Gata5*$^{+/-}$*Gata6*$^{+/-}$ (*D, H, L*) embryos. In wildtype, *Gata5*$^{+/-}$ and *Gata6*$^{+/-}$ embryos (*A-C*, *E-G*), the aortic valve opens in the left ventricle. In *Gata5*$^{+/-}$*Gata6*$^{+/-}$ embryos, the aortic valve opens in the right ventricle (*D, H*), leading to double outlet right ventricle (DORV). (*I-K*) Transverse section through the heart showing an intact ventricular septum in wildtype, *Gata5*$^{+/-}$ and *Gata6*$^{+/-}$ embryos. In *Gata5*$^{+/-}$*Gata6*$^{+/-}$ embryos (*L*), a membranous ventricular septal defect (VSD) is observed, which is associated with the DORV (arrow). Bars = 300μm (*A-D* and *I-P*) and 100μm (*E-H*). AV, aortic valve; LV, left ventricle; RV, right ventricle.

Figure 3.8. Modulation of gene expression in *Gata5*$^{+/-}$*Gata6*$^{+/-}$ embryos. Q-PCR analysis performed on hearts of E12.5 embryos. Transcript levels are normalized to GAPDH used as internal control in WT and *Gata5*$^{+/-}$*Gata6*$^{+/-}$ embryos. Expression of *Gata5* and *Gata6* is reduced by 40-50% and there are no significant changes in the levels of *Gata4* mRNA. Note the downregulation of *BNP*, *Myh6*, *Nkx2.5* and *Tbx20* transcripts and the increased expression of *Raldh2* mRNA (* $P<0.05$, $n = 6$ for WT and $n = 4$ for *Gata5*$^{+/-}$*Gata6*$^{+/-}$ embryos).

Table 3.1. Summary of the cardiac phenotypes of the *Gata4/Gata5* double heterozygotes at E15.5. MV, mitral valve; TV, tricuspid valve, VSD, ventricular septal defect.

Table 3.2. Summary of the cardiac phenotypes of the *Gata5/Gata6* double heterozygotes at E15.5. VSD, ventricular septal defect.

A

Age	No. Genotyped	WT	Gata4$^{+/-}$	Gata5 $^{+/-}$	Gata4$^{+/-}$;Gata5$^{+/-}$
P30	71	28 (24%)	11 (15%)	28 (39%)	4 (6%)
E17.5-E18.5	30	8 (27%)	9 (30%)	6 (20%)	6 (20%)
E14.5-E15.5	107	28 (26%)	26 (24%)	33 (30%)	19 (18%)
E11.5-E12.5	75	14 (23%)	20 (27%)	18 (24%)	18 (24%)

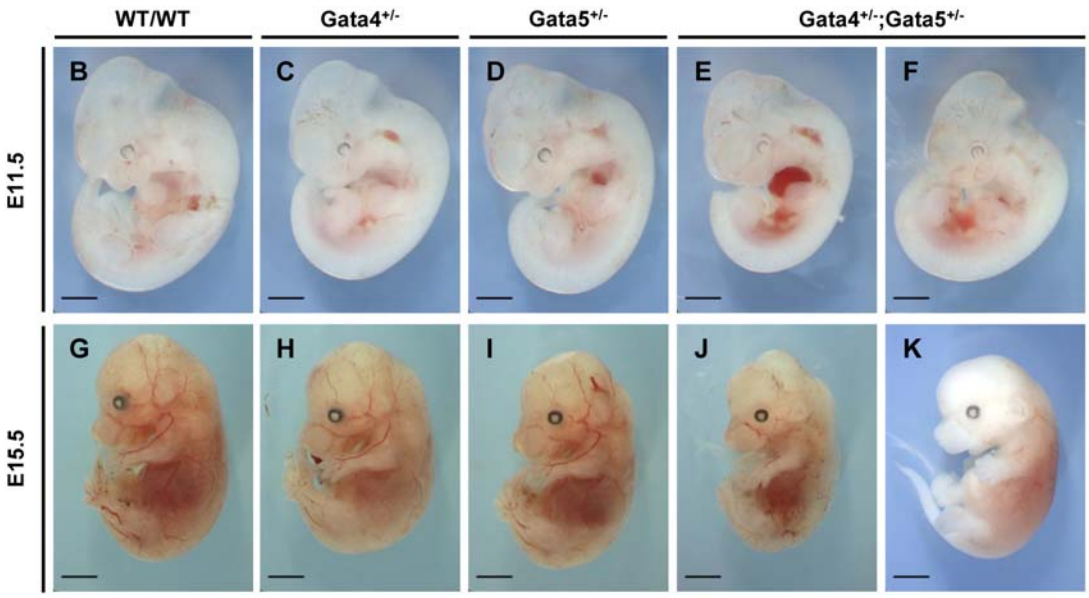


Figure 3. 1. Reduced viability of *Gata4*$^{+/-}$*Gata5*$^{+/-}$ heterozygotes

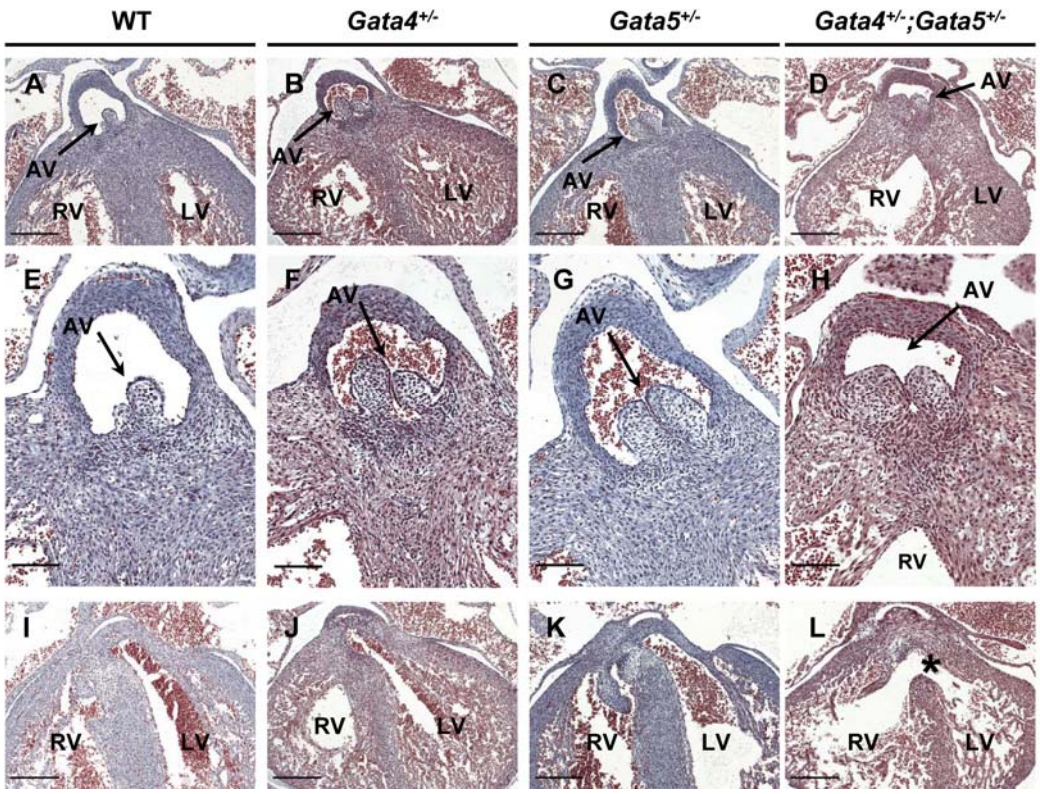
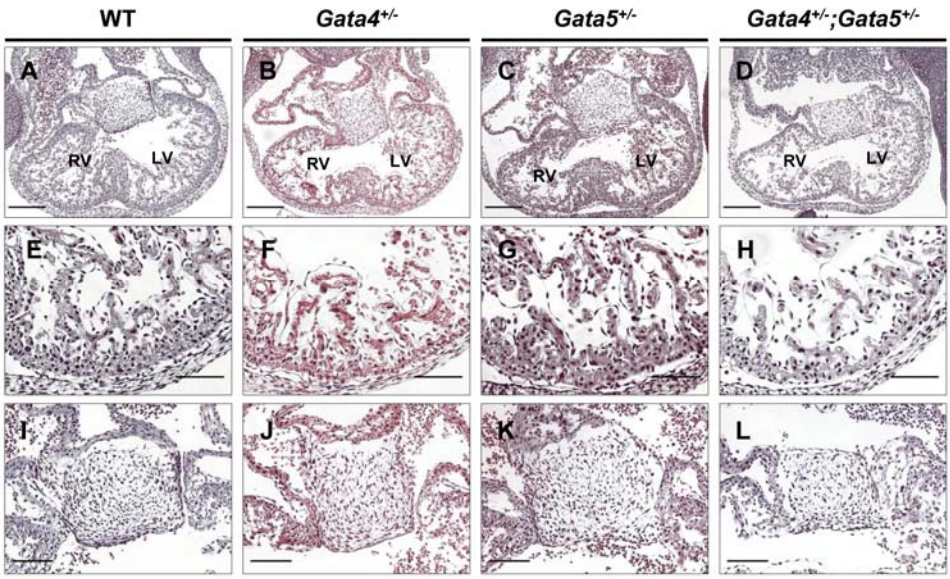


Figure 3. 2. Cardiac defects in *Gata4*$^{+/-}$*Gata5*$^{+/-}$ heterozygotes



M

Number	WT	Gata4$^{+/-}$	Gata5$^{+/-}$	Gata5$^{+/-}$Gata4$^{+/-}$
Myocytes in LV	333,06 ± 17,04	209,5 ± 17,68 ***	324,92 ± 10,72	209,67 ± 7,38 ***
Endocardial cells in LV	126,5 ± 5	114,42 ± 4,88	126,25 ± 5,13	109,24 ± 2,77 *
Cushion cells in AVC	273,47 ± 23	199,33 ± 18,90 *	242,53 ± 28,53	158,5 ± 10,16 **

Figure 3. 3. Ventricular wall and endocardial cushion defects in *Gata4$^{+/-}$Gata5$^{+/-}$* embryos

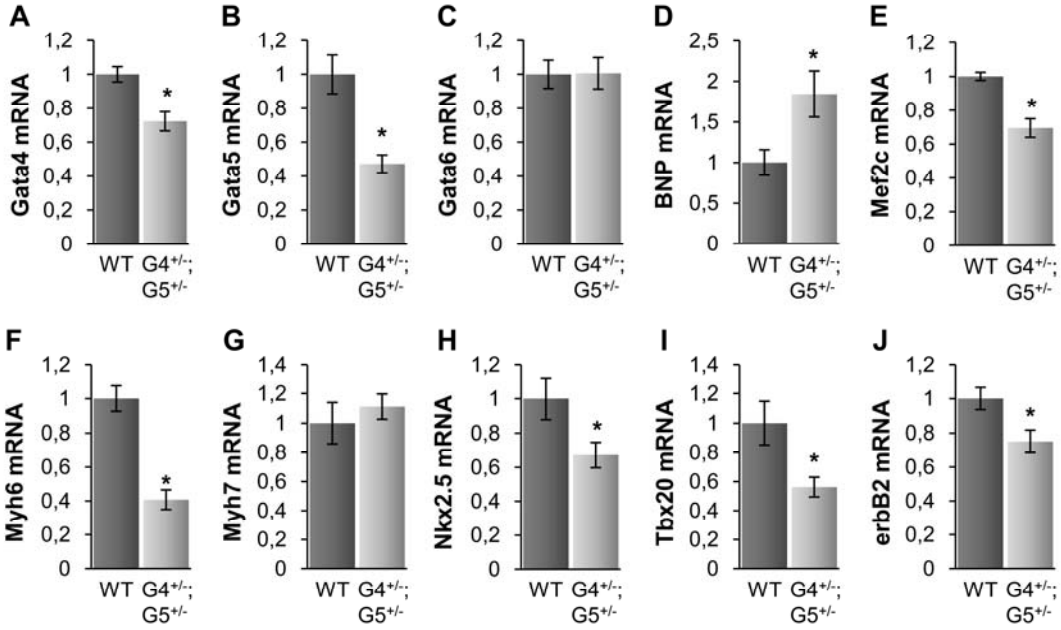


Figure 3. 4. Modulation of gene expression in *Gata4*$^{+/-}$*Gata5*$^{+/-}$ embryos

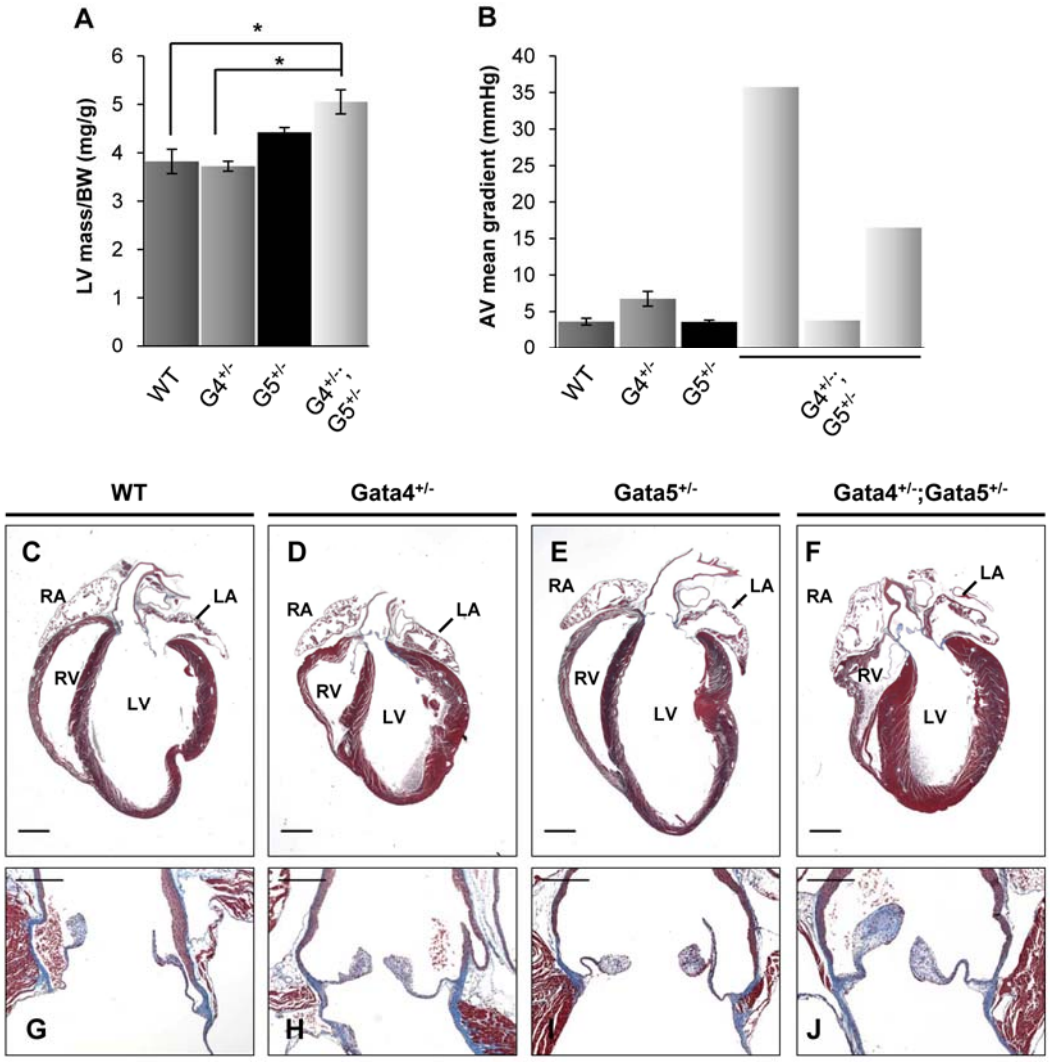


Figure 3. 5. Aortic stenosis in adult *Gata4*$^{+/-}$*Gata5*$^{+/-}$ heteroztgotes

A

Genotype	No. Genotyped	WT	Gata5$^{+/-}$	Gata6$^{+/-}$	Gata5$^{+/-}$;Gata6$^{+/-}$
P30	99	26 (26%)	54 (55%)	18 (18%)	1 (1%)
P0-P1	105	37 (35%)	26 (25%)	20 (19%)	11 (10%)
E18.5	91	22 (24%)	23 (25%)	30 (32%)	14 (15%)
E14.5 - E15.5	53	13 (25%)	12 (23%)	17 (32%)	9 (17%)
E11.5-E12.5	68	14 (21%)	15 (22%)	13 (19%)	23 (33%)

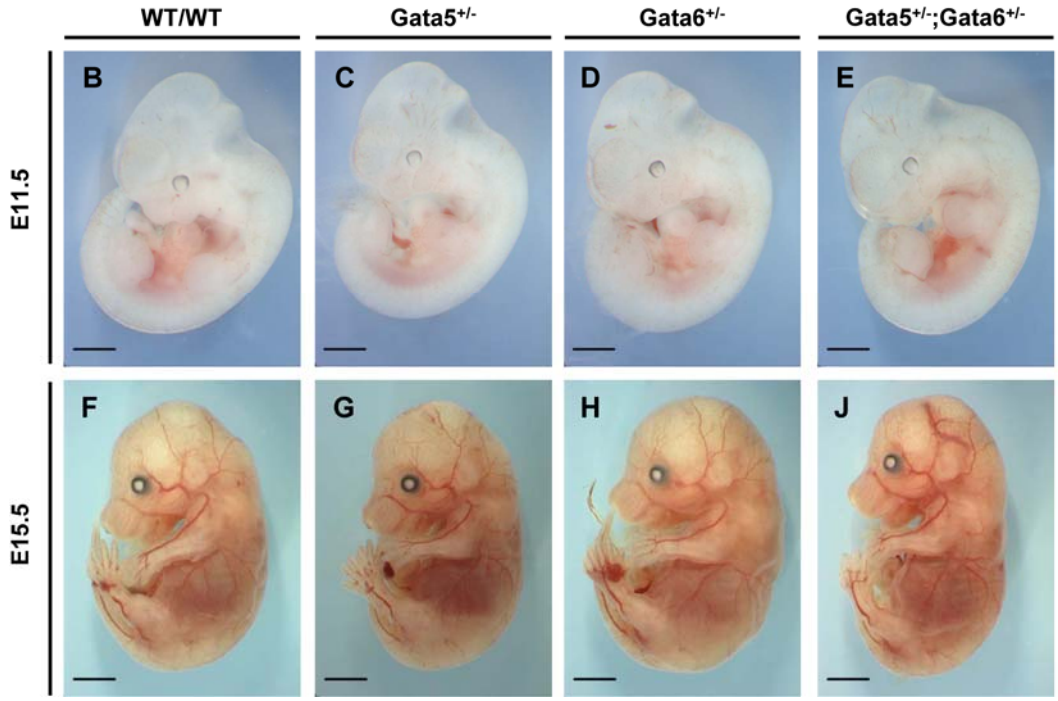


Figure 3. 6. Reduced viability of *Gata5*$^{+/-}$*Gata6*$^{+/-}$ heterozygotes

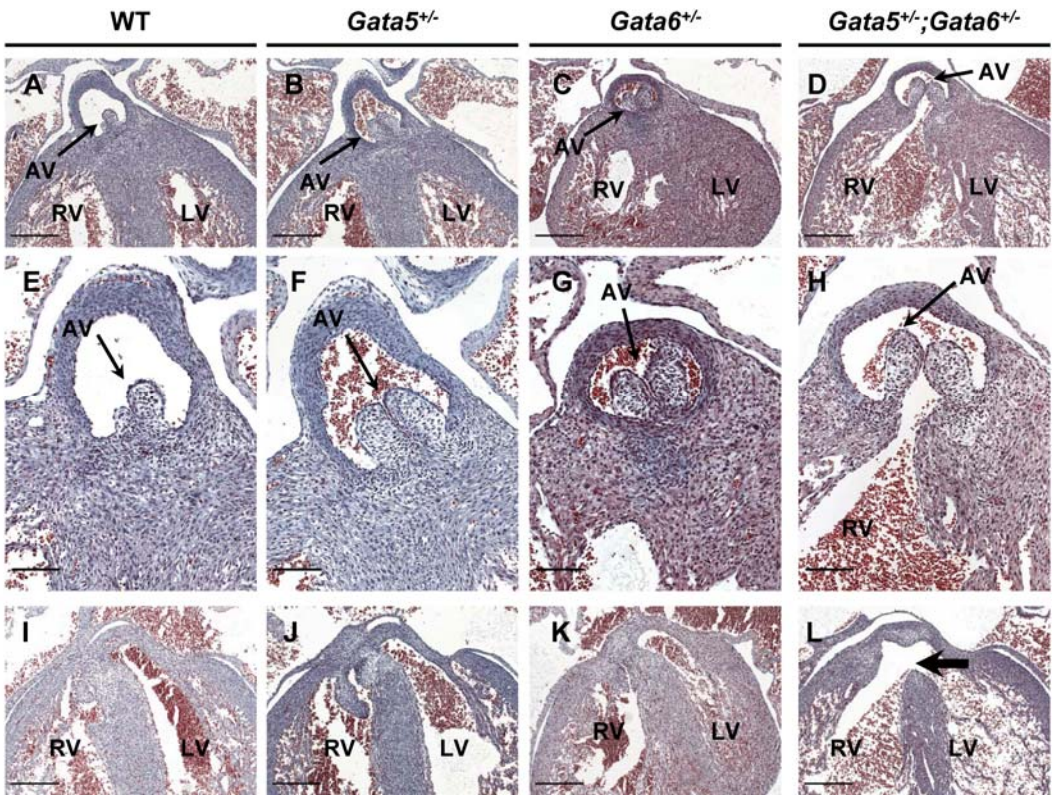


Figure 3. 7. Cardia defects of *Gata5*$^{+/-}$*Gata6*$^{+/-}$ heterozygotes

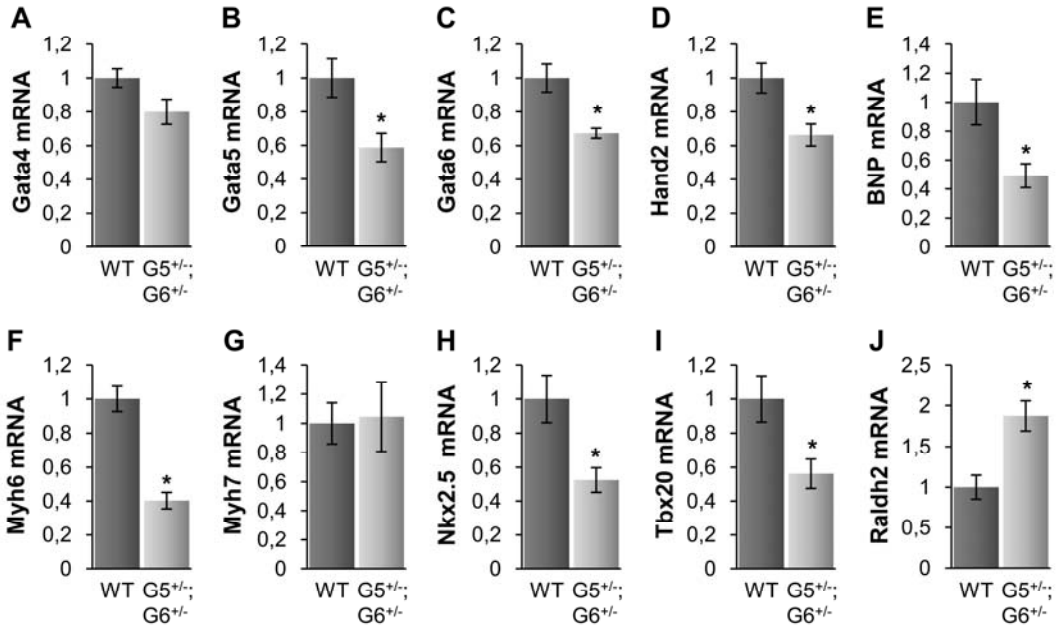


Figure 3. 8. Modulation of gene expression in *Gata5*$^{+/-}$*Gata6*$^{+/-}$ embryos

Phenotype	WT	Gata4$^{+/-}$	Gata5$^{+/-}$	Gata5$^{+/-}$Gata4$^{+/-}$
Normal	1	3/7	1	1/7
Double outlet right ventricle	0/5	2/7	0/5	6/7
Small VSD	0/5	3/7	0/6	1/7
Large membranous VSD	0/5	2/7	0/5	5/7
Atrioventricular septal defect	0/5	1/7	0/5	2/7
Thick MV and TV	0/5	2/7	0/5	4/7
Myocardial thinning	0/5	4/7	0/5	5/7

Table 3. 1. Summary of the cardiac phenotypes of the *Gata4/Gata5* double heterozygotes at E15.5

Phenotype	WT	Gata5$^{+/-}$	Gata6$^{+/-}$	Gata5$^{+/-}$Gata6$^{+/-}$
Normal	5/5	5/5	5/5	1/7
Double outlet right ventricle	0/5	0/5	0/5	6/7
Membranous VSD	0/5	0/5	0/5	6/7
Thick myocardium	0/5	0/5	0/5	5/7

Table 3. 2. Summary of the cardiac phenotypes of the *Gata5/Gata6* double heterozygotes at E15.5

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Chapter 4

Discussion

GATA5, a member of the GATA family of zinc finger proteins is expressed in a highly specific and evolutionary conserved manner in vertebrate hearts. The spatio-temporal pattern of GATA5 in endocardial cells and endocardial cushion of the AV canal and OFT suggest a specialized function in cardiac morphogenesis. The *in vivo* experiments performed during this thesis, and presented here, clearly demonstrate the requirement of GATA5 during heart development since loss of *Gata5* leads to bicuspid aortic valve (BAV), the most common CHD in humans. Despite its importance, our understanding of the mechanisms underlying BAV formation is still ambiguous and the *Gata5* null mice provide an interesting model to study the development of this CHD. Of note, this is the first study that reports the requirement of endocardial cells in this process as evidenced by the presence of BAVs in mice lacking endocardial *Gata5*. Moreover, this work shows that genetic interactions between the cardiac GATA factors are essential for proper heart development. We found that GATA5 interacts with GATA4 and GATA6 for proper OFT rotation and for valve development. In what follows, the role of GATA5 in heart development will be discussed and a more global discussion on valvular diseases will be presented; lastly, the potential role of GATA5 in transcriptional control of endocardial development will be reviewed.

4.1. GATA5 and development of an experimental model of BAV

4.1.1 Clinical importance of BAVs

BAV disease has been grossly underestimated over the years as it remains mostly asymptomatic during childhood and early in even adulthood. However, clinicians are increasingly aware of the frequency of BAV and its importance as a risk factor as well as its role in cardiac and aortic complications. In fact, BAV is said to account for more morbidity and mortality that all other CHDs combined (445). This high morbidity and mortality can be attributed to a multitude of complications associated with BAV, including aortic stenosis, regurgitation, infective endocarditis and aortic complications such as dilatation, dissection and rupture.

Aortic stenosis is the most common complication, occurring in about 50% of individuals. The progression of stenosis parallels the development and progression of the sclerotic changes in the aortic valve. Indeed, severely stenosed BAV are very rigid

because of fibrosis and calcification, which occurs in a similar fashion to that seen in patients with tricuspid aortic valve calcification. Beppu S *et al* demonstrated that stenosis progresses more rapidly if the cusps are asymmetrical in size and in the anterioposterior position (792). Similarly, in children, aortic valve disease has a more rapid progression in patients with fusion of the right and non-coronary leaflet (449). Studies also revealed that patients with stenosis secondary to BAV require valve replacement one decade younger than those with tricuspid aortic stenosis.

BAV is also associated with dilatation of the aortic root and ascending aorta, which is observed in 50-60% of patients. Aortic root dilation is further presumed to be a precursor of aortic rupture and dissection, which are fatal events. Dilatation of the aorta has been characterized by degeneration and fragmentation of the elastic fibers, loss of smooth muscle cells, increase in collagenous fibers and replacement of the degenerated tissue with interstitial basophilic cells (793;794). It has been shown that the ascending aorta can be dilated whether the valve is stenotic, incompetent or normal (795). Moreover, even after replacement of the aortic valve, patients with BAVs show progressive dilatation of the proximal ascending aorta (796). These finding suggest that a structural weakness of the aortic wall is already present and several potential mechanisms have been proposed, including deficient fibrilin-1 content and increased matrix metalloproteinases (793;797).

Although BAV may remain undetected for a long period of time, the majority of patients will require surgery at some point in their life. At present, there is no generally accepted pharmacological treatment for aortic stenosis. However, a large number of patients with BAV develop calcification and statins have been shown to inhibit calcification in cultured porcine aortic valve myofibroblasts by inhibiting the cholesterol biosynthetic pathway (798). Moreover, recent studies demonstrate that statin treatment in patients with calcific aortic valve may slow the rate of progression of stenosis, decrease calcium accumulation and delay the degeneration of the bioprosthetic valve (799;800). In patients with Marfan syndrome, characterized by skeletal anomalies (long thin extremities and loose joints), dislocation of the lens and aortic dilatation, β-blocker therapy has proven beneficial for slowing the rate of aortic root dilatation, thus reducing the development of aortic complications (801). There is evidence that beta-blockade might reduce shear stress and limit matrix remodelling, thus decreasing elasticity of the aortic wall (802). Whether this treatment is useful in retarding aortic dilation and

decreasing the risk of aortic dissection is unknown; however β-blockers are prescribed to BAV patients with moderate aortic dilatation.

4.1.2 Genetic network involved in BAV formation

Deciphering the molecular events involved in normal valve development or BAV formation is critical for elucidating the molecular basis of BAV, which remains poorly understood. Identification of GATA5 as critical for formation of a normal aortic tricuspid valve and the finding that its loss causes BAV will help in molecular valve dissection of valve development To date, only a few genes have been associated with BAV in humans or in mouse models. One of the first mouse models of BAV was reported with targeted deletion of the *NOS3* gene where 40% of *NOS3* null mice have BAVs (459). This suggested that this signalling pathway was critical for valvulogenesis. More recently, it has been reported that the BAVs in *eNOS*$^{-/-}$ mice result from the fusion of the right and non-coronary leaflet (R-N) (451). It was hypothesized that this was due to a defect in the formation of the OFT endocardial cushions, providing one of the first clues into the potential mechanism leading to BAV formation. NOS3 expression is restricted to endocardial cells in the heart and is shear-stress dependent (803). The formation of the endocardial cushions depends on the EMT process, which is also shear stress dependent. This led to the hypothesis that *NOS3* deficiency might alter endocardial cell migration during EMT, causing anomalous development of the valve cushion. Similarly, the *Gata5* null mice have a prevalence of 26% of BAVs and show the same type of fusion of the aortic valve leaflets as in the *eNOS*$^{-/-}$ mice. Since both of these genes are expressed in endocardial cells and in the OFT, it would be interesting to cross mice heterozygous for *NOS3* and *Gata5* and investigate the possible genetic interactions in aortic valve development. In addition, scanning of the *NOS3* locus revealed the presence of GATA binding sites and my studies have demonstrated that GATA5 is able to activate the *NOS3* promoter, suggesting that it could be a downstream effector. Thus, there is a possibility that both genes act in the same pathway. To get more insight into this hypothesis, QPCRs or whole-mount ISH on embryonic *Gata5* null hearts could be performed to determine if the *NOS3* transcripts levels are altered.

BAVs were also observed in a small proportion of mice haploinsufficient for *Nkx2.5*, which has been associated with CHDs in humans (289). Nkx2.5 is a key regulator of cardiac development and inactivation of *Nkx2.5* is lethal, which has

prevented the analysis of its role in aortic valve development. In human BAV, no mutations have been reported for Nkx2.5. In the last decade, *Nkx2.5* has been shown to modulate the ECM of the aorta during cardiovascular development through regulation of collagen type I (804). During endocardial cushion formation and EMT, the ECM supports proliferation and migration of newly transformed mesenchymal cells into the cardiac jelly. At this stage, there is a low abundance of collagen, which reflect the architecture required for the matrix function (805). However, as valvulogenesis progresses, there is an increase in collagen deposition in the ECM, which is required to provide stiffness and strength to the mature valve leaflet (806). Moreover, mutations in collagen type 1 have been linked with the Ehlers-Danlos syndrome, which is characterized by skin and bone abnormalities as well as mitral and aortic valve dysfunction (807). In the study reported by Ponticos *et al*., a GATA-like binding site was mapped near the NKX binding site. They proposed a model whereby GATA6 and Nkx2.5 synergize leading to transcriptional activation of *Col1a2* in response to activation of VSMCs. Moreover, studies from our lab have demonstrated that Nkx2.5 and GATA5 are able to synergistically activate the ANF promoter, suggesting that they may physically interact (124). All of the observations suggest that Nkx2.5 and GATA5 could interact together to regulate *Col1a2* expression in the heart and that *Col1a2* is a possible downstream target of GATA5. Similarly to NOS3, it would be of interest to see if the GATA5 and Nkx2.5 transcriptional cascades converge together, resulting in an increased severity of BAVs when mice heterozygous for each gene are crossed together.

To date, only mutations in human *Notch1* have been associated with BAVs (453;455). Garg V *et al* identified a nonsense and a frameshift mutation in two families with BAV and valve calcification, which provided compelling evidence that *Notch1* haploinsufficiency was a cause of aortic valve disease. Notch1 is expressed in the endocardium and OFT cushion mesenchyme consistent with the aortic valve phenotype in these patients. In the same study, it was demonstrated that Notch1 as well as the downstream target genes Hey1 and Hey2 were able to inhibit Runx2 activity, suggesting that the Notch signalling prevents calcium deposition. The Notch pathway plays major role in multiple developmental processes, including cardiovascular development (808). Moreover, it has been shown to be critical for EMT that contribute to the heart valves and is highly expressed in the valve mesenchyme and endocardium.

In mammals, the Notch pathway is composed of four type I transmembrane receptors (Notch1-4) and 5 type I transmembrane ligands, including JAG1. Mice lacking *JAG1* die at E10.5-E11.5 with defects in yolk sac and embryonic vasculature (809). More recently, conditional deletion of *JAG1* in the SHF has demonstrated its requirement in aortic arch remodelling and septal development (810). As these mice die perinataly, it has not been possible to analyse the morphology of the aortic valve. I have demonstrated that JAG1, which is expressed in the endocardial cells in the same temporal window as GATA5, is strongly downregulated in *Gata5* null and *eGata5*$^{-/-}$ mice, suggesting that endocardial GATA5 is upstream of JAG1 and regulates its expression. Mutations in human JAG1 have been associated with Alagille syndrome, which is an autosomal dominant disorder characterized by skeletal, ocular, renal, heart and hepatic defects (811). One of the principle findings in Alagille syndrome is CHD, characterized by right-sided OFT defects, pulmonary stenosis and TOF. In addition, *JAG1* mutations have also been associated with TOF and pulmonary stenosis (812;813). This phenotype is consistent with the expression pattern of JAG1 in the endothelium, neural crest cells and smooth muscle cells of the pulmonary arteries (814;815). It is interesting to note that decreased JAG1 expression could lead to reduced Notch1 activation and subsequent Notch signalling in the OFT, which could contribute to abnormal endocardial cushion formation and fusion of the aortic valve leaflets. Immunohistochemical staining of embryonic hearts with an antibody against *Notch1* NCID could answer the question regarding downregulation of the Notch1 intracellular domain in *Gata5* null mice. Scanning of the *JAG1* locus reveals a number of potential GATA binding sites, conserved across species, 3 Kbp upstream of the ATG as well as about 20Kbp upstream, suggesting that a distal GATA dependent enhancer might exist, regulating expression of JAG1. Today, the regulatory region of JAG1 has not been determined but we could perform transfection experiments on these potential regulatory regions to answer this hypothesis.

4.1.3 Endocardial GATA5 expression is necessary for normal aortic valve development

The goal of this thesis was to evaluate the role of GATA5 in cardiac morphogenesis in mammals. Eventhough GATA5 was shown to play a major role in cardiac development in *zebrafish*, the first report on the inactivation of this gene in mice revealed no major cardiac defects (33;757). However, the strategy used by targeted

the first exon only, resulting in deletion of the first 157 aa with the possibility to form a truncated protein containing both zinc fingers and C-terminal activation domain. Characterization of the *Gata5* locus revealed the presence of two distinct isoforms of GATA5 in chicken and mammals, with one lacking the entire exon two (first zinc finger) (759). This N-terminal truncated protein retains the ability to bind DNA and activate target genes (365;760). Based on these observations, the role of GATA5 in heart development cannot be definitely determined based on this mouse model.

We therefore decided to generate a new *Gata5* null allele by deleting the second zinc finger and C-terminal activation domain, thus ensuring deletion of both isoforms. Analysis of the aortic valve in these mice revealed the presence of BAVs with a prevalence of 26%. In the endocardial cushions of the OFT, there is a contribution of endocardial-derived, SHF-derived and neural crest-derived mesenchyme. To gain more insight into the role of GATA5 in endocardial development and which cell type contributed to these defects, we generated a conditional mouse model lacking GATA5 in endothelial cells (*eGata5*$^{-/-}$). In the *eGata5* mutant mice, a 70% reduction in GATA5 transcripts was observed and attributed to its reduction in endocardial cells as GATA5 is more abundantly expressed in endocardial cells. We noticed a prevalence of 21% of BAVs in *eGata5*$^{-/-}$ mice, which is similar to *Gata5* null mice. Interestingly, this reflects a cell autonomous function for GATA5 in regulating endocardial cushion development.

In both mouse models, the BAV resulted from the fusion of the right and non-coronary leaflet (R-N). This is the same type of fusion as was observed in *NOS3*$^{-/-}$ mice (451). Previous studies have demonstrated that fusion of the leaflets is an early event during valvulogenesis and is the key factor in formation of a BAV (450). This suggests that each leaflet of the aortic valve acquires its configuration before the end of valvulogenesis. Based on the study performed by Fernandez *et al*, it has been suggested that defective migration of mesenchymal cells could be responsible for fusion of the leaflets. Collagen explants cultures have been useful to determine some of the genes involved in EMT. In this case, it would be complicated by the fact that most mesenchymal cells have migrated into the cushions and thus, the experiment could not be conclusive. However, it would be possible to look at the OFT endocardial cushions at E9.5 when the neural crest mesenchyme has not reached the proximal part of the OFT. We calculated the number of cells within the OFT cushion and noticed a reduction in Gata5 *null* compared to control embryos. Immunohistochemical studies

using proliferation and migration markers could be performed at E9.5-E10.5 to assess if this is due to a migration or proliferation defect.

As substantial complications are associated with BAV, both mouse models represent a powerful tool to study the evolution of the disease. We have not observed aortic root dilation or calcification of the aortic valve by 200 days but as mice got older, some started to die prematurely. Autopsy of a dead female *Gata5* null mouse revealed massive hypertrophy as well as the presence of a thrombus in the left atrium, which is often linked with valve disease. In addition, one out of six (17%) old *Gata5* null mice with BAV, which had a peak aortic valve gradient of 96 mmHg (8 mmHg in controls) showed signs of aortic regurgitation. This is similar to complications that arise in humans with BAV, where aortic regurgitation accounts for 1.5-3% of cases (442).

4.2. Identification of novel genetic interactions between the GATA factors

Genetic predisposition to CHD has been clearly established but in most CHDs complex inheritance and partial expressivity has complicated human genetic studies aimed at identifying disease causing genes. They have also suggested the presence of genetic modifiers but few have been identified. A number of transcription factors, whose mutations are linked with CHDs, including NKX2.5, GATA4, and TBX5, have been shown to act cooperatively to regulate normal heart development. Genetic interactions between Tbx5 and GATA4 were found important in atrial septum development (127;291). Moreover, Olson and colleagues have shown that cooperative interactions between GATA4 and GATA6 are important for proper septation of the OFT as well as formation of the compact myocardium (777) Overall, these observations suggest that combinatorial interactions between transcription factors play a critical role during embryonic cardiac development. This is consistent with the phenotype that was observed in the *Gata4/Gata5* and *Gata5/Gata6* double heterozygotes generated during my studies. Compound heterozygosity of *Gata4* and *Gata5* resulted in perinatal lethality and mutants embryos displayed cardiac defects including DORV, VSD, CAVC and hypertrophied AV valves. Only 6% of *Gata4/Gata5* double heterozygote embryos survived to adulthood and these had aortic stenosis. Compound heterozygosity of *Gata5* and *Gata6* also resulted in DORVs, which was associated with a subaortic VSD. These results strongly suggest that GATA5 interacts with the other cardiac GATA factors for proper formation and rotation of the OFT.

Members of the GATA family are highly related within their DNA binding domain but display more divergence outside this region. The overlapping expression patterns of GATA1, GATA2 and GATA3 in some hematopoietic cells suggest that these factors may have redundant functions. However, there is increasing evidence suggesting that a given GATA protein does not compensate for the absence of the other and that they interact together for normal hematopoietic development. Of note, *in vitro* hematopoietic differentiation of *Gata1*$^{-/-}$ ES cells leads to a 50-fold increase in *Gata2* mRNA in erythroid cells, however, these cells are arrested in differentiation and die by apoptosis (816). The blood islands of double *Gata1*$^{-/-}$*Gata2*$^{-/-}$ embryos are devoid of primitive erythroid cells in contrast to *Gata1*$^{-/-}$ or *Gata2*$^{-/-}$ embryos, suggesting that they cooperatively interact in hematopoiesis (817). The knockin of *Gata3* into the *Gata1* locus partially rescues the *Gata1* null phenotype with increased survival of erythroid precursor cells and increased survival up to E13.5 (818). This observation also suggests that each GATA factor has a distinct function in development. In addition, when the *Gata2* and *Gata3* transgene are under the control of *Gata1* regulatory elements, rescue of the embryonic lethal phenotype of the *Gata1* mutation is observed, suggesting that the hematopoietic GATA factors are not functionally equivalent (819). The similar expression of GATA2 and GATA3 in the central nervous system (CNS) again indicates possible genetic interactions. No expression of GATA3 could be detected in the CNS of *Gata2*$^{-/-}$ embryos, suggesting that expression of GATA3 is dependent on GATA2 (689).

The ability to interact with each other is also well conserved among the cardiac GATA factors. Experiments from our lab demonstrated that GATA4 and GATA6 heterodimerize and synergistically activate the *Nppa* and *Nppb* promoters in cardiomyocytes, providing the first evidence of physical and functional interactions (723). Xin *et al* demonstrated that GATA4 and GATA6 interact in outflow tract development (777). The *Gata4/Gata6* compound mutants displayed VSDs, PTAs and myocardial hypoplasia. These defects were distinct from the *Gata4* and *Gata6* heterozygote mice, clearly showing that both genes cooperate together in cardiovascular development. Another study reported that both GATA4 and GATA6 are required for heart development as their inactivation results in *acardia* in mice (778). These authors noticed that cardiac myocyte differentiation was altered in these embryos while formation of the SHF progenitor cells was normal. It is worth noting that

in zebrafish, Holtzinger *et al* demonstrated that zebrafish depleted of both *Gata5* and *Gata6* (*gata5+6* morphants) are completely heartless, which is distinct from depletion of *Gata5* or *Gata6* alone, indicating that both proteins are essential for cardiomyocyte development (820). In addition, zebrafish depleted of both *Gata4* and *Gata6* were shown to display an early block in liver development and lack liver buds, indicating that they interact together for liver growth and not only in heart development (783). Interactions among the GATA factors are important for heart and liver development in *Xenopus*. This was shown by co-injection of *Gata4* and *Gata6* morpholinos, which resulted in a reduction of heart and liver precursors and to *cardia bifida* (821). Overall, the function of GATA factors seems to be conserved across species and the genetic interactions are important to allow normal development of the heart, liver and possibly other organs where they are co-expressed.

4.2.1. Interaction of GATA factors in outflow tract development

The studies performed during this project demonstrated the requirement of two GATA factors in OFT development. In both cases, the compound heterozygotes had abnormal development of the OFT, leading to a DORV. DORVs arise when there is an arrest in the rotation of the OFT, resulting in the presence of both great vessels leaving the right ventricle. Previous studies have revealed that all three cardiac GATA factors are expressed in the SHF while GATA6 is also expressed in neural crest cells. In light of the results obtained in chapter 2.2, it is not possible to say which cell type is responsible for the DORV. Thus, it would be interesting to generate compound heterozygotes in which GATA4 and GATA5 expression is deleted only in the SHF, by using the *Mef2*^{Cre} transgenic mouse. In the case of GATA6, two compound mutants could be generated, one in which GATA6 is deleted in the neural crest and one where GATA6 is inactivated in SHF cells. This could also give us some insight into their role in the SHF as well the molecular pathways affected by their loss. Recently, Rojas *et al* reported that deletion of *Gata4* in the SHF resulted in VSDs, ventricular hypoplasia and myocardial thinning (718). However, the authors did not observe OFT defects as were shown in other mouse models of GATA4. Their hypothesis was that GATA5 and GATA6 might compensate for GATA4 in the SHF. Generating double heterozygotes in the SHF would certainly answer this question. Presently, it is still unclear if the presence of GATA6 in the neural crest was able to influence the number of SHF cells in these mutants.

The cardiac OFT is formed and subsequently remodelled by complex interactions between the endocardium, myocardium and CNC cells. Endocardial cells will undergo EMT, which contributes to the formation of the OFT cushions. The SHF cells populate the OFT cushions and the CNC cells migrate into the distal OFT cushions, where they are required for septation and proper alignment of the OFT. Based on the expression pattern of GATA4 and GATA5 in the heart, it is possible that reduced expression of these two GATA factors in the endocardium may influence rotation and alignment of the OFT, which could explain the DORV phenotype observed in *Gata4/Gata5* mutant embryos. To verify this hypothesis, we have crossed our *Tie2*^{cre}+;*Gata5*$^{WT/Flox}$ mice with *Gata4*$^{WT/Flox}$ mice to delete both genes in endocardial cells. In addition, reciprocal interactions between the SHF and neural crest have been shown to be important for proper addition of SHF cells to the OFT (319). Since the OFT cushions are composed of three cell types, it is highly possible that reciprocal interactions between SHF and neural crest with endocardial cells occur within the cushions. As mentioned before, GATA6 is also expressed in the neural crest and there is a possibility that it could influence the number of endocardial cells in the cushion, their proliferation or survival. This could be tested by crossing heterozygous mice which lack *Gata6* in neural crest cells with mice heterozygote for *Gata5* and scoring for the DORV phenotype. To get better insight into the mechanism leading to DORV, TUNEL staining should be performed to assess if cell death is present in the OFT. Moreover, proliferation as well as migration markers should be used to stain the OFT cushions and determine if misalignment of the OFT is due to defective migration or proliferation of endocardial, neural crest or myocardial cells. In addition, candidate QPCR analysis of dissected outflow tracts at E9.5-E10.5, when the endocardial cushions form and the OFT aligns, could be performed to find downstream targets of the GATA factors in the OFT. Downregulation of these potential target genes in the OFT could possibly contribute the defective alignment of the OFT.

4.2.2. Interaction of GATA factors in valve development

Previous studies have suggested a role for GATA4 in endocardial cells. Of note, deletion of *Gata4* in the endocardium using the *Tie2*^{cre} transgenic mice resulted in embryonic lethality with hypoplastic cushions, suggesting that GATA4 is required for growth of the cushions and EMT (719). As discussed in the introduction, VEGF plays an important role in valve development, where it is required for the proliferation of

endocardial cells. Based on the observation that a number of GATA binding sites are present on the human VEGF promoter, we hypothesized that GATA4 could regulate its activity. Interestingly, I have shown that GATA4 is able to transactivate the VEGF promoter as well as cooperatively regulate its activity in presence of Tbx5 or Tbx20. A point mutation in GATA4 (G295S) strongly reduced its DNA binding activity as well as cooperative interactions with the T-box factors. This is consistent with the related G296S mutation in mice, which was shown to reduce GATA4 DNA binding activity as well as physical interactions with Tbx5 (127). In addition, GATA4 stable clones were generated in TC13 cells, which is a mesodermal cell line that can be differentiated into endothelial cells upon treatment with RA (368). Proliferation assays indicated that the GATA4 stable clones were proliferating more rapidly compared to controls, thus bringing more evidence for a proliferative role for GATA4 in endocardial cells.

The generation of *Gata4/Gata5* double heterozygotes allowed to test the interactions between these two proteins in endocardial development. We noticed that the 70% of AV valves were hypertrophied and 15% of embryos had a common AV valve, which results from abnormal remodelling of the cushion. The number of cushion cells was also reduced compared to control, which could highly contribute to these defects. These cardiac abnormalities could be the results of defective expression of these proteins in endocardial cells or in the myocardium as myocardial-endocardial signalling is important for endocardial cushion formation. To answer this question, we have crossed our $Tie2^{cre}$+;$Gata5^{WT/Flox}$ mice with $Gata4^{WT/Flox}$ mice to delete both genes in endocardial cells.

Two thirds of surviving *Gata4/Gata5* double heterozygotes presented high aortic mean pressure gradient as well as left ventricular and aortic valve hypertrophy, similar to humans with aortic stenosis. It would be interesting to follow up these mice to see the evolution of the disease with aging, particularly regarding the rigidity or calcification of the aortic valve, which is often associated with aortic valve disease. The phenotype in these mice is more severe than in the *Gata5* null mice, strongly indicating that both genes interact together in normal endocardial development. As deletion of *Gata4* in the endocardium is lethal embryonically, it has not been possible to analyse the morphology of the aortic valve. The increased aortic mean pressure gradient in these mice is similar to that of *Gata5* null mice that have BAVs, which suggest that the double

heterozygotes could have congenital BAVs. We are currently looking at this possibility by examining the morphology of the aortic valve in newborn pups.

As discussed in the introduction, multiple signaling pathways are required for proper development of the cardiac valves. In this study, we demonstrated a strong downregulation of Tbx20. Tbx20 is expressed in both myocardial and endocardial cells where it is required for proliferation and differentiation (550;551). Moreover, *neuromancer*, the Tbx20 ortholog in *Drosophila*, has been shown to be a potential downstream target of *pannier* in the heart (822). This function appears to be conserved with mice and we observed multiple GATA binding sites on the putative murine Tbx20 promoter, further suggesting that the GATA factors may cooperatively regulate Tbx20. It would be worthwhile to test this hypothesis in transfection and chromatin immunoprecipitation assays. Moreover, co-immunoprecipitation studies could be undertaken to test for physical interactions between GATA4 and GATA5. Based on the physical interactions that were demonstrated between GATA4 and GATA6 from our lab and the fact that this requires the zinc finger domain which it is highly similar among GATA factors, it seems reasonable to hypothesize that GATA4 and GATA5 as well as GATA5 and GATA6 may physically interact.

CHD is the leading cause of infant morbidity and mortality in the world. Significant advances towards understanding the molecular mechanisms involved in CHD have been made in the last decade. Eventhough family studies led to the discovery of mutations of cardiovascular developmental genes such as Tbx5, Nkx2.5 and GATA4, the majority of cases of CHD have no identified cause or association. CHD has an increased risk of recurrence within families and among the affected relatives, the cardiovascular phenotype usually differs, occurs with incomplete penetrance and variable expressivity, thus suggesting that modifying factors, including genetic and environmental influences, are at play (440). Based on these observations, understanding the molecular and genetic mechanisms involved in cardiac morphogenesis and CHD would offer valuable insight that would lead to a better diagnosis and prevention of serious cardiac complications with age. The results obtained during this study strongly suggest that GATA5 may be a good candidate CHD causing gene. Future studies aimed at elucidating downstream effectors of GATA5 in endocardial cushion development will provide a better understanding of the molecular mechanism involved in BAV as well as other valve diseases. Moreover,

characterization of the genetic interactions between cardiac GATA factors as well as the genetic pathways involved in OFT development will also contribute to a better understanding of the interactions between the different cells types in the OFT endocardial cushions and formation of DORVs.

4.3. Valve diseases progressively lead to degenerative phenotypes

In addition to BAVs, other valve disease also tend to degenerate over time, leading to similar complications. Valvular heart disease (VHD) is a common condition that increases with age, with a prevalence of 13% in individuals 75 years of age or older (823). Defective development of the heart valves occurs in 20-30% of all CHDs, with an incidence of congenital valve defect estimated to be as high as 5% of live births (439;475). The extracellular matrix plays a mechanical role during valvulogenesis and is rich in hyaluronan, versican and fibronectin, which facilitate cell migration, growth and dynamic morphological changes that give rise to the heart. The mature valve structure is composed of ECM, valvular interstitial cells (VIC) and overlying endothelial cells. In addition, the ECM is composed of three highly organized layers that are primarily composed of collagens, proteoglycans and elastin. There is increasing evidence that loss of ECM organization is associated with changes in mechanical properties, leading to dysfunction in adult valve disease. Consistent with this, studies of diseased valves in adult patients have shown thickening of the valve leaflets, collagen fiber disorganization, increased VIC density and calcification (824-826).

Periostin is a secreted protein that promotes adhesion and migration and is expressed in the embryonic cardiac valves in addition to cancer cells, vascular smooth muscles cells, fibroblasts and wound-site blood vessels (827;828). A number of studies have demonstrated a requirement of periostin for normal cardiac valve development and its critical role in cardiac valve maturation (828-830). Of note, the valve leaflets of *periostin*$^{-/-}$ mice are hypertrophied and shortened by 3-months of age and the tendinous cords of the AV valves are either truncated or missing. The phenotype of *periostin*$^{-/-}$ mice is similar to the degenerative changes seen in prolapsed human mitral valves or BAVs (828;830). More recently, it was reported that infants with congenital BAV stenosis have reduced valve periostin expression, suggesting that reduced expression of periostin during development may result in congenital valve malformations (830). Whether periostin played any pathophysiologic role in adult valve disease remains

unclear. In human atherosclerosis and rheumatic valve disease periostin levels were pathologically overexpressed in infiltrated inflammatory cells and myofibroblasts in areas of angiogenesis (831). In addition, periostin was able to form tubes and mobilize endothelial cells in the mid-region of the valve, especially where the normal structure of the valve endocardium is disrupted. Based on these observations, it is possible that periostin enhances the recruitment of circulating endothelial progenitor cells or the penetration of microvessels from the annulus region into the leaflet of the valve.

Elastin, a component of elastic fibers, is and EMC protein required for tissue integrity and mobility (832). Mutations in elastin have been identified in patients with supravalvar aortic stenosis (833). Moreover, elastin haploinsufficiency results in cardiovascular abnormalities in 10-45% of patients with valve problems (834). Based on these observations, it was hypothesized that elastin haploinsufficiency in mice would result in viable aortic valve disease and hence the function of elastin during progressive degeneration of valve disease could be assessed. Hinton R et al showed that the *Eln*$^{+/-}$ mouse model leads to a progressive aortic valve malformation (835). Interestingly, by the adult stage, the *Eln*$^{+/-}$ aortic valve became elongated, thinned, stiff, elastic fibers were decreased and fragmented and collagen bundles were disoriented. Moreover, the authors noted a decrease in TGFβ signaling and echocardiographies revealed the presence of regurgitation and aortic stenosis, suggesting that the incidence of disease increased over time. Thus, the elastin haploinsufficient mice proved to be a useful model for improving our understanding of valve disease pathogenesis.

Versican, another EMC component, plays important roles during cardiac development and also in adult cardiovascular diseases (193;836). Increasing evidence has revealed that versican cleavage occurs throughout cardiac development by members of the ADAMTS family (837). Among them, *Adamts9* is expressed in derivatives of the SHF, vascular smooth muscle cells, the arterial wall, mesenchymal cells of the valves and non-myocardial cells of the ventricles, suggesting it may play an important function during cardiac development and in the adult heart as well (838). *Adamst9* haploinsufficiency leads to abnormal thickening of the semilunar valve leaflets as well as increased proteoglycan content in the aortic valve. In addition, the mitral valves show disorganized fibrous components of connective tissue as well as increased versican accumulation. These observations suggest that gradual accumulation of uncleaved versican may be the underlying cause of the anomalies.

Previous studies have suggested that the mechanism of aortic valve degeneration is similar to that underlying atherosclerosis. The process includes the destruction of the endothelial layer of the valve, invasion of inflammatory cells into the valve, accumulation of low density lipoprotein, proliferation of valvular interstitial cells, ECM remodelling and eventually calcification of the valve (826). Interestingly, MMPs and tissue inhibitor of matrix metalloproteinases, including MMP-3, MMP-9 and TIMP-1, have been found to be increased in calcified human aortic valves. In addition, Fibulin-4, another component of the ECM, has been shown to play an important function in maintaining TGFβ signal and ECM integrity. Loss of *fibulin-4* leads to aortic valve stenosis as well as ascending aortic aneurysm and attenuated TGFβ signaling (839). Other molecules such as BMP2, Sox9 and Notch signalling are involved in aortic valve calcification. Reduced *Sox9* function was shown to lead to increased expression of bone-related genes and activation of inflammation and ECM remodelling, leading to calcification of the valves (840). Notch1 signaling in aortic valve cells represses activation of osteoblast genes like runx2 through mediation of Bmp2 (453;841).

Based on these observations, it would be important to assess potential aortic valve and aortic wall degeneration in *Gata5* null old mice and determine which of these molecules are involved in valve pathogenesis resulting from loss of *Gata5* function.

4.4. Genetic networks underlying outflow tract defects

The incidence of conotruncal defects, which include PTA, DORV, TGA and TOF, is 12-14% of all CHDs (475). Significant advances in our understanding of how the cardiac OFT normally forms was provided by Kirby M and colleagues when they demonstrated that neural crest cells contribute to the septation of the OFT. Ablation of the neural crest results in a spectrum of cardiovascular defects including PTAs, outflow misalignments and defective remodelling of the great arteries (423). Based on previous studies in chick and human, it is well accepted that the OFT undergoes rotation during its remodelling (81;842). Moreover, it was discovered in the last couple of years that rotation of the myocardial wall of the OFT is required for normal positioning of the great arteries (843). Thus, complex and highly regulated cellular and morphogenetic events are required for proper septation and development of the OFT.

Persistent truncus arteriosus occurs when the conotruncal area does not divide properly during embryonic cardiac development into a separate aorta and pulmonary

artery, resulting in a common trunk. Mutations in *Tbx1* have been associated with PTAs in mice and humans (844). Loss of function of *Tbx1* in the mesoderm results in reduced cell proliferation in the pharyngeal mesoderm, which contributes to the septation, growth and alignment defects (845). Loss of genes that are normally expressed in the neural crest also leads to PTAs, including *Gata6*, *Sema3C*, *Pax3* and *Pitx2* (315). Transposition of the great arteries normally occur in 10-11% of children who have CHDs (461). In TGA, the pulmonary artery is supplied by the left ventricle and the aorta by the right ventricle instead of the opposite. This results in mixing of unoxygenated blood and cannot occur without the presence of a VSD, an ASD or a patent ductus arteriosus. In humans, mutations in *Zic3*, *Nkx2.5*, *Nodal* and *FoxH1* have been associated with TGA (429). These genes have all been involved in establishing left-right patterning during embryonic development, suggesting that mutations in laterality genes could result in this defect. Consistent with this, it has been shown that *Pitx2c* null mice display TGA, in addition to DORV (846). Double outlet right ventricle represent a continuum of CHDs where the aorta originates from the right ventricle instead of the left and is always associated with a VSD. The VSD can be subaortic, subpulmonary, not committed to any vessel or doubly committed (underlying both vessels). DORVs have been reported in association with chromosome 22q11 deletion and attributed to loss of *Tbx1* function (847). This phenotype is also observed in mouse knockouts for *TGFβ2*, *Gata4*, *Pitx2*, *Sox4*, *Dvl 2*, *Pax3*, *Cx40* and in mice defective in the retinoic acid signalling pathway (847).

A genetic network, involving the SHF and neural crest, has been revealed to be crucial for OFT formation. Mice lacking *Isl1* die embryonically and display absence of right ventricle and OFT, which are derived from the SHF (44). Importantly, Isl1 is not expressed in the OFT and right ventricle but is detected in the SHF and is considered as one of the earliest markers of the SHF (44). Other genetically engineered mice show similar cardiac phenotypes. Inactivation of *Mef2c* results in failed looping with a single ventricular chamber and defective OFT (304). More recently, it was demonstrated that *Mef2C* is a direct transcription target of Isl1 and GATA4 in the anterior heart field, suggesting that the GATA factors and Isl1 are among the earliest transcription factors controlling OFT development (305). Foxh1 also plays an important role in the formation of the RV and OFT as mice lacking FoxH1 lack a RV and have reduced expression of early cardiac markers including *Mef2c*, *Hand2*, *Fgf8* and *Tbx5*

(306). When Hand2 was deleted in neural crest cells, VSDs, DORV and aortic arch arteries defects were observed (848). More recently, it was suggested that Hand2 influences OFT development by regulating genes related to cell migration, proliferation/cell cycle and intracellular signalling, including *Cx40*, *Hey1*, *Foxc1*, *ADAM19*, *Col11a1* and *NFATc2*. Thus, expression of Hand2 in neural crest cells may be required for proper patterning of the OFT, generation of an appropriate number of neural crest-derived cells for elongation of the OFT and cardiac cushion formation (849).

The role of GATA factors in the SHF has not been studied in detail to date. However, there is a reinforcing circuit in the SHF between GATA factors and Nkx2.5. Interestingly, Molkentin *et al* described the analysis of a *Gata6* enhancer that is restricted to the pharyngeal mesoderm, OFT and right ventricle at E9.5 in the mouse (788). This enhancer was directly activated by Nkx2.5 and thus, it appears that GATA factors and Nkx2.5 can cross-regulate one another's expression. Here, we showed that Nkx2.5 was strongly downregulated in the embryonic hearts of the compound heterozygotes, consistent with the ability of GATA factors to regulate Nkx2.5 expression. Interestingly, mutations in *Nkx2.5* were found to be among the most common monogenic loci associated with DORV in humans (847). Therefore, reduction of *Nkx2.5* expression could contribute to the DORV phenotype observed in the double GATA het mice. Moreover, a strong reduction in *Mef2c* expression was also observed in the double heterozygotes and as GATA4 is able to regulate a *Mef2c* enhancer, this could also contribute to the defects observed in these mice. Similarly, *Mef2c* was shown to be downregulated in the *Gata4/Gata6* compound heterozygotes, which also contributed to the defects observed in these mice (777). The *Gata4/Gata6* double heterozygote embryos displayed PTAs as well as ventricular hypoplasia. As discussed in this section, PTAs and DORVs result from defective septation or misalignement of the OFT. Thus, alteration in the levels of any 2 cardiac GATA factors leads to OFT defect, suggesting that a threshold of GATA factors is required for proper OFT development. Based on the pattern of expression of GATA6 in the heart, it is likely that defective *Gata6* expression in the neural crest may underlie the PTA phenotype as neural crest are essential for septation of the OFT. A recent study reported that the myocardium at the base of the OFT is involved positioning of the great arteries (843). In both the *Pax3*$^{-/-}$ and *Pitx2c*$^{-/-}$ embryos, the authors showed that abnormal expression of the *y96-Myf5-nlacZ-16* (*96-16*) transgene, which marks the myocardium at the base of the OFT, resulted in

arrested rotation of the OFT. As GATA4 and GATA6 are both detected in the myocardium of the OFT, there is a possibility that abnormal expression of these genes at the base of the pulmonary trunk may lead to arrested rotation of the OFT in the *Gata4/Gata6* or in our double heterozygote embryos. Moreover, as GATA4 and GATA5 are highly expressed in the endocardium, it is likely that this cell type may also underlie the OFT defects. We showed that the endocardial cushions of the *Gata4/Gata5* double heterozygotes are smaller and have a reduced number of mesenchymal cells. As the endocardial-myocardial signaling is important for heart development, it is possible that a reduction in the number of OFT cushion cells may affect the number of myocardial cells at the base of the OFT trunk, thus leading to arrested rotation of the OFT. In order test these hypotheses, future studies aimed at verifying the expression of neural crest, endocardial, myocardial and SHF markers should be undertaken by whole mount ISH in *Gata4/Gata5* and *Gata5/Gata6* embryos. We could also cross mice heterozygous for *Gata4*, *Gata5* or *Gata6* with other mouse models that lead to DORVs in order to determine if they converge or act in the same pathway in OFT development.

4.5. GATA5 in transcriptional control of endocardial development

Endocardial cells are required for multiple aspects of cardiac morphogenesis, including valve and septal development. They are also required, through cross-talk via secreted factors, in the division of the truncus arteriosus into the aortic and pulmonary trunks and formation of the trabecular myocardium. Given the high number of CHDs that are represented by valvular abnormalities, septal defects, cardiac conduction defects and ventricular noncompaction, understanding endocardial development is very important clinically. However, numerous questions regarding the origin and development of endocardial cells have still not been answered although recent studies have hinted at the existence of a common progenitor with myocardial cells. Thus, a more detailed understanding of the molecular mechanisms that regulate endocardial development is necessary in order to have a better insight into human CHDs and in the hope of developing preventive and therapeutic strategies.

In the last decade, attention has been directed towards the myocardial-endocardial signaling and its importance in cardiac morphogenesis. Formation of the trabecular myocardium is essential to heart development and adult cardiac function.

However, the molecular regulation of this process is still poorly understood. Endocardial cells play an important role in modulating cell proliferation and morphogenesis of trabecules. Few pathways, including Neuregulin/ErbB, EphrinB2/EphB4 and Bmp10, have been shown to be involved in this process. The *NRG1*$^{-/-}$, *ErbB2*$^{-/-}$ and *ErbB4*$^{-/-}$ embryos die embryonically with failure of cardiac development due to lack of trabeculation (145-147). NRG1 is expressed in the endocardium whereas the ErbB receptors are expressed in the myocardium. Bmp10 is expressed in the trabecular myocardium and loss of Bmp10 leads to embryonic lethality, which is associated with decreased ventricular trabecular proliferation (148). Lastly, ephrinB2 and ephrin receptor B4 are both expressed in the ventricular endocardium and deficiency in either of them leads to failure of myocardial trabeculae formation (850). The Notch signaling pathway has emerged, in the last couple of years, as an important player in formation of the ventricular trabecular network. Of note, Notch activity can be detected in trabeculae in the earliest developmental stages with expression in the endocardium (851). The trabeculation defective phenotype of *Notch1* and *RBPJ*κ mutants strongly supports a role in the development of the ventricular myocardium (149;851). Moreover, Notch mutant embryos show defective expression of Bmp10, ephrinB2 and NRG1, which are essential for trabeculation. Based on these studies, the authors have proposed a model where Notch1 acts directly on the ephrinB2/ephB4 pathway, which is required for NRG1 production and subsequent activation of ErbB2/ErbB4 receptors in the myocardium. Notch1 is also able to activate Bmp10 in the myocardium in a non-cell autonomous manner in order to maintain proliferation of the trabecular cardiomyocyte population.

GATA5 is expressed in the endocardial cells around the trabecules from E9.5-E12.5, suggesting that it may play a role in trabeculae formation. In the *Gata5* null embryos, we observed reduced ventricular trabeculation and upon QPCR analysis, we noticed reduced expression of *NRG1*, *erbB2* and *ephB4* transcripts, which are involved in trabeculation. This highly suggest that GATA5 acts upstream of these genes to regulate proper formation of ventricular trabecules. Based on the Notch model, we can hypothesize that GATA5 directly regulates the ephrinB2/ephB4 pathway, which then activates the NRG1/ErbB pathway or that GATA5 directly activates all of these genes independent of one another. As of yet, the specific interaction between *Gata5* and these genes is still unknown. Whole mount ISHs or immunohistochemical studies will

be required to pinpoint the exact location and confirm that these genes are downregulated in the endocardium of the *Gata5* null mice. It will be interesting to verify the expression of *Notch1* and *Bmp10* in these embryos to see if GATA5 regulates. At the same time, it would be important to get a hold of the *NRG1*, *ErbB2*, *Notch1*, *Bmp10* and *ephB4* deficient mouse models or collaborate with these groups to test the expression of GATA5 in these embryos and get a better understanding of the relationship between these genes and where exactly GATA5 comes to play in this hierarchy.

Endocardial cells play a critical role in valvulogenesis and a subtle alteration in the formation of the endocardial cushions may lead to valve defects. As discussed in section 1.1.7.4 of the introduction, a number of signalling pathways are required for valve development. In addition, a number of transcription factors, including NFATc1, Twist1, Tbx20 and Sox9, have also been shown to play important functions during valvulogenesis. NFATc1 belong to the NFAT family of transcription factors. *NFATc1* null mice die at E14.5 due to lack of cushion growth and remodelling (272;273). EMT occurred normally in these embryos, suggesting that NFATc1 was required at later stages of valve development. The heart defects were rescued upon endothelial-specific expression of NFATc1, demonstrating that the requirement of NFATc1 expression in endocardial cells for proper valvulogenesis (280). Further studies on the *NFATc1*$^{-/-}$ embryos as well as avian endocardial cushions demonstrated that NFATc1 is required for endocardial cell proliferation and induction of Cathepsin K, which is expressed in the remodelling valve leaflet (852). Earlier this year, it was reported that NFATc1 is expressed in human calcific aortic valves, providing evidence that dysregulation of NFATc signaling leads to progressive valve disease (853). These studies suggest that NFATc1 is required for remodelling during valve development.

Sox9 is a SRY-related transcription factor that has been shown to be important for sex determination and cartilage formation. In the mouse, Sox9 is expressed in the precursor cells of the endocardial cushions and its expression is maintained in the adult valve leaflets (854). Loss of *Sox9* leads to embryonic lethality with hypoplastic endocardial cushions that fail to complete EMT, suggesting a role in the early stages of valve development (855). In addition, *NFATc1* was found to be mis-expressed in the mutant embryos, which suggest that the endothelial delamination process during EMT did not occur properly. Moreover, endocardial specific deletion of *Sox9* leads to

embryonic lethality by E14.5 and mutants embryos displays hypoplastic cushions, reduced cell proliferation and altered ECM deposition, indicating a role in expansion of the valve progenitor pool (854). Recently, heterozygous loss of *Sox9* in the *Col2a1*^{Cre} lineage was show to result in thickened valve leaflets that progressively develop calcific lesions, thus identifying Sox9 as a potential candidate for calcific valve disease (840).

Twist1, which belong to the class II basic helix-loop-helix transcription factor, promotes cell proliferation and migration of embryonic progenitor cells and transformed tumour cells (856). In humans, *Twist1* haploinsufficiency has been associated with the Saethre-Chotzen syndrome, characterized by craniofacial defects as well as CHD (857). In the mouse, Twist1 is expressed in the endocardial cushions of the AVC and OFT during valve development while its expression is downregulated in the remodelling valve leaflet. Loss of *Twist1* in mice leads to embryonic lethality by E11.5 and embryos display abnormal migration of neural crest cells, hypoplastic limb buds and vascular defects (858). However, initial stages of valve development seemed normal but analysis of the remodelling valves was precluded due to the early lethality. In the last couple of years, Yutzey and colleagues have shown that Twist1 is required in endocardial cushions to promote cell proliferation and migration by increasing expression of *Cadherin 11*, *periostin* and *Mmp2* while repressing the differentiation marker *aggrecan* (551). In the same study, it was also reported that Twist1 can induce *Tbx20* expression, which was also shown to promote proliferation of endocardial cushion cells. Moreover, persistent expression of Twist1 in the remodelling valves led to increased valve cell proliferation, increased *Tbx20* and EMC gene expression, consistent with their previous study (859). In human diseased aortic valves, expression of *Twist1* was increased and cell proliferation was observed near nodules of calcification, suggesting that Twist1 may be involved in the progression of valve diseases (859).

Tbx20, a member of the T-box family, is expressed in the endocardial cushion mesenchyme during embryonic development. Mutations in *Tbx20* have been linked to ASDs as well as valve defects, suggesting that it may play an important role during valvulogenesis (555). Mice lacking *Tbx20* die at E10.5, which precluded analysis of valve development in these mice. To elucidate the role of Tbx20 in valve development, gain and loss of functions were performed in avian endocardial cells. Like Twist1, Tbx20 promotes cell proliferation and migration as well as expression of *Mmp9* and

Mmp13 while repressing *aggrecan* and versican (550). Furthermore, Bmp2 treatment of avian endocardial cells resulted in increased *Tbx20* expression while loss of *Tbx20* led to increased *Tbx2* gene expression. Thus, Tbx20 seems to promote cell proliferation of the mesenchymal valve precursor cells and repress ECM remodelling.

All of these transcription factors are expressed in endocardial cells or endocardial cushions at the same time as GATA5, suggesting that they could act in the same pathway or genetically interact in valve formation. As I have described in chapter 2, we found a strong downregulation of *Tbx20* in the *Gata5* null embryos, suggesting that it may be a downstream effector of GATA5 in the endocardium. It would be interesting to verify the expression of these transcription factors in *Gata5* null embryos to see if they could act as downstream effectors of GATA5 in valve development. Moreover, it is highly possible that we may find GATA binding elements in the promoter of these genes as GATA binding sites are found in practically all promoters. Transfection as well as chromatin immunoprecipitation experiments could be undertaken to determine if these genes are direct targets of GATA5 in endocardial cells.

It has been hypothesized that the embryonic AV canal contains the precursors of the AV node of the CCS. This suggest that formation of the endocardial cushions is critical to AV node development. The first hint for a role for GATA5 in the CCS came from electrocardiographic analysis of old *Gata5*$^{-/-}$ (450 days) mice. We observed a prolongation in the PR interval, which is associated with AV node (first degree AV block) dysfunction. Our hypothesis is that GATA5 may regulate the expression of genes in endocardial cushions that become confined to the CCS. In general, markers expressed in the embryonic AVC become restricted to the AV node, AV valves encircling myocardium of the AV junction and anterior node. For example, *Tbx3* is expressed in the AV canal during embryonic development where it act as a repressor and its expression becomes further restricted to the AV node and small parts of the right AV junction (166). Consistent a role for GATA5 in the CCS, we noticed a significant downregulation of *Cx40* (25%), *Cx43* (25%) and *HCN4* (34%) transcripts already in embryonic *Gata5* null hearts. It will be important to perform whole mount ISH or immunohistos between E9.5 and E11.5, during growth and formation of the AV canal endocardial cushions, to pinpoint the exact location where these genes are downregulated. Moreover, immunohistochemistry staining of embryos at later stages of AV node development will be required to verify the expression of these markers in the

CCS. To get better insights into the downstream effectors of GATA5 in the CCS, QPCR analysis, using dissected AV canals from E9.5-E12.5 *Gata5* null mice, should be performed. Candidate genes, including *Cav1.3*, *Tbx2*, *Tbx3*, *Bmp2*, *Msx2*, which have been shown to play important roles in the AVC, should be tested. Lastly, further electrophysiology studies should be performed to detect at what age the conduction defects appear.

4.6. Conclusion and perspectives

The studies presented in this thesis helped define and the role of GATA5 during endocardial development and cardiac morphogenesis. They clearly showed that GATA5 is required for proper formation of the aortic valve and that loss of *Gata5* increases the risk of developing BAVs, which are the most common CHD in humans. Additionally, the discovery that BAVs developed due to defective endocardial GATA5 expression reflects a cell autonomous role for GATA5 in the endocardium for valve formation. This is the first time that a direct connection between BAV and the cell type involved in this malformation is made. In addition, my studies revealed cooperative interactions between GATA5 and the other GATA factors in OFT and valve development. The documented function of GATA5 in cardiac morphogenesis opens the door for further molecular studies of endocardial development and valve formation. They also provide rational genetic analysis that explore the link between GATA5 and CHD in cardiac development.

Because it was initially thought that GATA5 was not playing a critical role in the heart and hence, few studies addressed the GATA5 pathway in the mammalian heart. What are the downstream targets of GATA5 in the heart? A microarray performed with *Gata5* null and control heart would give us some insight into this question. Even more interesting would be to dissect AV canals and the OFT at E9.5-E10 to find specific target genes in endocardial cushions when EMT is happening. This would also help elucidate the genes and subsequently the molecular basis underlying BAV formation.

How is GATA5 regulated? Are there any enhancers governing its expression specifically in the endocardium versus myocardium and epicardium? This notion is consistent with previous studies in chicken where an enhancer was found directing expression of GATA5 in the endocardium. To get some insight on how GATA5 may be regulated in the mouse, a thorough look at the *Gata5* locus should be undertaken to

find regions where clusters of transcription factors could be located. Transfection as well as chromatin immunoprecipitation assays would be performed to assess the activity of these regulatory regions and if GATA5 directly binds these regulatory elements.

We noticed that *Gata5* null embryos had hypoplastic hearts while adult mice had mild left ventricular hypertrophy. This phenotype was not observed with endothelial specific inactivation of *Gata5*, suggesting that another cell type contributed to these defects. As GATA5 is expressed in a subset of myocardial cells, it is possible that the phenotype observed is due to lack of GATA5 in the myocardium. To test this hypothesis, *Gata5* could be inactivated in the *Nkx2.5*^{Cre} lineage. NKX2.5 is expressed in the myocardium at the earliest stages of cardiac development and it is possible that these cells could contribute to the *CMV*^{cre} phenotype. The role of GATA5 could be assessed in these mice by echocardiographic and electrocardiographic analysis as well as pressure overload experiments. Embryos would be dissected, similar to *Gata5* null mice, to assess if the hearts are hypoplastic during embryonic development and if hypertrophy develops in the adult. In addition, we could not assess conduction defects in old *Tie2*^{Cre}*+;Gata5*$^{F/F}$ mice as they did not reach that age yet. The question that arises is: are the conduction defects due to expression of GATA5 in endocardial cells or another cell type? If we find that old *Tie2*^{Cre}*+;Gata5*$^{F/F}$ mice have first degree AV block, this would confirm that expression of GATA5 in the endocardial cells is required for proper CCS function. If it turns out that these mice don't have conduction defects, then another cell type must contribute to the phenotype. It has been hypothesized that the AVC endocardial cushions are the precursors of the AV node. Interestingly, epicardial cells also produce mesenchymal cells that migrate to the AVC endocardial cushions, which suggest that the first degree AV block could be due to absence of GATA5 in these cells. Generation of mice with targeted deletion of *Gata5* in the epicardium, by using *WT1*^{Cre} transgenic mice, would answer this hypothesis.

The results obtained during this PhD strongly suggest that human GATA5 may be a candidate CHD causing gene. Mutations in *hGATA4* have been described for a long time in the literature. Eventhough GATA6 has been linked to PTAs in mouse models, it was only this year that three papers reported mutations in *hGATA6* in individuals with PTAs, tetralogy of Fallot and ASDs. No mutations in *hGATA5* have been reported yet in the literature but hopefully, some will be found in the near future.

Thus, screening for mutations in patients with BAV will help to make a direct association between GATA5 and BAV. Moreover, it would be interesting to screen for mutations in individuals with VSDs, common AV valves and DORV as these were the major cardiac abnormalities found in compound heterozygotes.

Overall, the studies described in this thesis support the presence of genetic modifiers in CHD, which likely explain the incomplete penetrance and variable expressivity of phenotypes associated with a specific mutation. Knowledge resulting from the characterization and identification of such modifier genes in the mouse will definitely be relevant to human disease as signaling pathways involved in cardiac morphogenesis are strongly conserved between species.

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