KLF2 IS REQUIRED FOR NORMAL MOUSE CARDIOVASCULAR DEVELOPMENT

Aditi Raghunath Chiplunkar
Virginia Commonwealth University

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KLF2 IS REQUIRED FOR NORMAL MOUSE CARDIOVASCULAR DEVELOPMENT

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

By

ADITI RAGHUNATH CHIPLUNKAR

Advisor: Dr. Joyce A. Lloyd

Professor, Vice Chair of Education

Department of Human and Molecular Genetics

School of Medicine
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Table of Contents

Acknowledgement ........................................................................................................... iii

Table of Contents ........................................................................................................... vi

List of Figures ................................................................................................................ vii

List of Tables ................................................................................................................ ix

Abstract ........................................................................................................................ x

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW ............................................ 1

CHAPTER 2: CHARACTERIZATION OF KLF2-/- MOUSE CARDIAC DEVELOPMENT
DEFECTS AND IDENTIFICATION OF CELLULAR MECHANISMS

Summary ......................................................................................................................... 31

Introduction .................................................................................................................... 32

Methods ......................................................................................................................... 35

Results ............................................................................................................................ 46

Discussion ..................................................................................................................... 60
List of Figures

Figure 1-1: Schematic diagram of C2H2 zinc finger motif

Figure 1-2: Phylogenetic relationship of the human KLF/SP proteins

Figure 1-3: Schematic representation of cardiac development

Figure 1-4: Anatomic overview of heart valve development

Figure 1-5: E9.5 FVB/N KLF2/- atrioventricular cushions have accumulated cells lining the AV canal

Figure 1-6: Electron microscopy of E9.5 FVB/N KLF2/- atrioventricular cushions shows abnormal endocardial cell morphology

Figure 1-7: Network of cardiac jelly components

Figure 1-8: Molecules regulating endocardial cushion EMT

Figure 2-1: AV endocardial cushion mesenchymal cell count

Figure 2-2: Dissection of E10.5 AV region

Figure 2-3: E9.5 FVB/N KLF2/- hearts show increased number of cells lining the AV canal and consequently a constricted AV canal, compared to WT

Figure 2-4: E10.5 FVB/N but not mix and C57BL/6 KLF2/- atrioventricular endocardial cushions are hypoplastic and disorganized

Figure 2-5: E10.5 KLF2/- AV cushions show decrease in number of mesenchymal cells

Figure 2-6: E10.5 FVB/N KLF2/- hearts have accumulated endothelial cells lining the AV canal

Figure 2-7: E10.5 FVB/N KLF2/- hearts have abnormal cardiac jelly composition

Figure 2-8: E10.5 FVB/N KLF2/- hearts show reduction in transformed mesenchymal cells
Figure 3-1: Heart Rate in E10.5 mouse embryos

Figure 3-2: Cardiac output and Ejection Fraction in E10.5 embryos

Figure 3-3: Descending Aorta Velocity in E10.5 embryos

Figure 4-1: Signaling pathways in cardiac development

Figure 4-2: KLF2 regulates cardiovascular genes

Figure 4-3: Cardiovascular genes are dysregulated in FVB/N KLF2-/- AV region

Figure 4-4: Cardiovascular genes that are not differentially expressed in WT and KLF2-/- AV region

Figure 4-5: Tie2-cre KLF2-/- AV regions have decreased expression of cardiovascular genes, similar to the traditional KLF2 KO

Figure 4-6: ChIP assays were performed on cells obtained from E10.5 WT AV canal and cushion regions

Figure 4-7: Cardiac genes directly and indirectly regulated by KLF2

Figure 5-1: Ablation of KLF2 and KLF4 leads to hemorrhaging.

Figure 5-2: Double KO Primary head vein lacks continuous endothelial layer and have reduced numbers of mesenchymal cells near primary head vein

Figure 5-3: Crown to rump length

Figure 5-4: E10.5 FVB/N KLF2-/- but not mixKLF2-/- embryos have erythroid cells outside of the dorsal aortas

Figure 5-5: Bar graph representing crown to rump lengths of E10.5 embryos from KLF2+/- KLF4+/- matings

Figure 5-6: At E9.5 ablation of KLF2 and KLF4 leads to cranial hemorrhaging
Figure 5-7: Double KO Primary head vein lacks continuous endothelial layer

Figure 6-1: Model representing the role of KLF2 in cardiac development through downstream targets Sox9, Gata4, Tbx5 and Ugdh in the FVB/N genetic background at E10.5
List of Tables

Table 1-1: Regulators of Cardiac Development

Table 2-1: Cellular composition of the heart during early development

Table 2-2: Number of embryos observed and expected from FVB/N KLF2+/− matings

Table 3-1: Cardiac Function parameters and method of calculation

Table 3-2: Cardiac Function Analysis of E10.5 embryos

Table 4-1: Primer sequences for candidate KLF2 target gene

Table 4-2: ChIP primer sequences

Table 4-3: Candidate KLF2 targets in cardiovascular development

Table 4-4: Location and sequence of the potential KLF2 binding sites in the Tbx5, Gata4, Sox9 and UGDH promoters

Table 6-1 Variation in KLF2−/− cardiac phenotype based on genetic background of the mice
ABSTRACT

KLF2 IS REQUIRED FOR NORMAL MOUSE CARDIOVASCULAR DEVELOPMENT

By: Aditi Raghunath Chiplunkar

Advisor: Dr. Joyce A. Lloyd
Professor, Vice Chair of Education
Department of Human and Molecular Genetics
School of Medicine

Krüppel-like factor 2 (KLF2) is expressed in endothelial cells in the developing heart, particularly in areas of high shear stress, such as the atrioventricular (AV) canal. KLF2 ablation leads to myocardial thinning, high output cardiac failure and death by mouse embryonic day 14.5 (E14.5) in a mixed genetic background. This work identifies an earlier and more fundamental role for KLF2 in mouse cardiac development in FVB/N mice. FVB/N KLF2−/− embryos die earlier, by E11.5. E9.5 FVB/N KLF2−/− hearts have multiple, disorganized cell layers lining the AV cushions, the primordia of the AV valves, rather than the normal single layer. By E10.5, traditional and endothelial-specific FVB/N KLF2−/− AV cushions are hypocellular, suggesting that the cells accumulating at the AV canal have a defect in endothelial to mesenchymal transformation (EMT). E10.5 FVB/N KLF2−/− hearts have reduced glycosaminoglycans in the cardiac jelly, correlating with the reduced EMT. However, the number of mesenchymal cells migrating from FVB/N KLF2−/− AV explants into a collagen matrix is reduced considerably compared to wild-type, suggesting that the EMT defect is not due solely to
abnormal cardiac jelly. Echocardiography of E10.5 FVB/N KLF2-/− embryos indicates that they have abnormal heart function compared to wild-type. E10.5 C57BL/6 KLF2-/− hearts have largely normal AV cushions. However, E10.5 FVB/N and C57BL/6 KLF2-/− embryos have a delay in the formation of the atrial septum that is not observed in a defined mixed background. KLF2 ablation results in reduced Sox9, UDP-glucose dehydrogenase (UGDH), Gata4 and Tbx5 mRNA in FVB/N AV canals. KLF2 binds to the Gata4, Tbx5 and UGDH promoters in chromatin immunoprecipitation assays, indicating that KLF2 could directly regulate these genes. Thus KLF2 plays a role in EMT, through its regulation of important cardiovascular genes. E10.5 FVB/N KLF2-/− embryos show gaps in the endothelial lining at the dorsal aorta and a number of blood cells localized outside the aorta suggesting either hemorrhaging or inability of the hematopoietic progenitors to reach the aortic endothelium and enter circulation. This is not observed in KLF2-/− embryos in a mixed genetic background. In conclusion, KLF2-/− cardiovascular phenotypes are genetic background-dependent. KLF4 is another member of the Krüppel-like transcription factor family phylogenetically close to KLF2. It is known to play an important role in vascular regulation. Our studies show that in vascular development KLF4 plays a complementary role to KLF2, indicated by cranial hemorrhaging in E9.5 KLF2-/−KLF4-/− embryos in an undefined mixed background. This phenotype is absent in either of the single knockouts. The role of KLF2 and KLF4 in vascular development has not been studied as much as adult vascular regulation. This study begins to define the roles of these two transcription factors in development of blood vessels.

Congenital heart and valve defects are a common cause of infant mortality. KLF2 has never been studied in this context. Thus this work is important for a better understanding of the biology of valve development.
Mammalian Krüppel – like Factor (KLF) family

The members of the Krüppel–like factor family are DNA binding proteins that direct expression of a number of genes. They are named after the Drosophila protein Krüppel because of the presence of a characteristic carboxy-terminal DNA binding domain. The carboxy-terminal DNA binding domain consists of three tandemly repeated zinc fingers. These zinc fingers are of the classical C2H2 type and bind a zinc ion between two cysteine and two histidine residues in a tetrahedral configuration (Fig. 1-1). Both the fingers and the linking regions between the fingers are highly conserved within the family. The linker is a 7 amino acid inter-finger spacer TGEKP(Y/F)X. This structure is stabilized by zinc ion binding and the hydrophobic residues near the zinc binding site (Michael, Kilfoil, Schmidt, Amann, & Berg, 1992; Wolfe, Nekludova, & Pabo, 2000). The DNA binding domain is highly conserved within the KLF family. The KLF family thus recognizes similar DNA sequences in the promoters of the target genes. The amino-terminal domains of the KLF family members differ, thus recognizing and binding a large range of co-activators and co-repressors. There is spatial and temporal control on the transcription of KLF family members. While a number of the members are ubiquitously expressed, others are expressed in a small range of tissues. For example, KLF2 is expressed in lung, T-cells, erythroid cells, pre-adipocytes and endothelial cells, whereas KLF1 is expressed only in erythroid cells. The consensus KLF binding sequences are CC(A/G)CCC and GC-rich sequences in the promoter regions of their target genes.

Seventeen mammalian KLF family members have been identified and are named from KLF1 to KLF17 based on the Human Gene Nomenclature Committee convention. Research has
suggested that certain KLFs have very similar DNA binding sequence and overlapping roles. A
phylogenetic analysis of the KLF family identifies a number of subgroups based on sequence
similarities (Fig. 1-2). For example, KLF1, KLF2 and KLF4 are phylogenetically related and thus
are members of a subgroup. These 3 proteins are 90% similar in their zinc finger regions and
have overlapping roles in erythroid and vascular development.

Figure 1-1: Schematic diagram of C2H2 zinc finger motif. The two conserved cysteine
and histidine domains are shown in yellow and blue respectively, binding the zinc ion. The
top part is an enlarged diagram of one zinc finger and the bottom shows three tandemly
repeated zinc fingers. The linker sequence combining adjacent zinc fingers is shown in
green. Shown in orange are the hydrophobic residues required for stability of the structure.
The black residues are not structurally important but are important for DNA binding
(Knight,R.D. 2001).
Figure 1-2: Phylogenetic relationship of the human KLF/SP proteins. BLAST analysis of the NCBI non-redundant and high-throughput databases with the KLF1 protein sequence identified 21 unique human proteins with homology to KLF1, which were used for the analyses. The phylogram was drawn using the GROWTREE program in the GCG Wisconsin package. The *Drosophila melanogaster* Krüppel (Dm Krüppel) protein, which represents the ancestral form of the human KLF/Sp family, was used to root the tree (Zhang, P. 2005).
KLF2 expression and function

Among other cell types, KLF2 is expressed in erythroid and cardiovascular endothelial cells during development. KLF2 gene expression during development increases in response to increasing fluid shear forces, as shown in the chicken embryo (Groenendijk, Hierck, Gittenberger-De Groot, & Poelmann, 2004). Its expression correlates with shear forces in mouse development (Lee et al., 2006). Fluid shear stress is the frictional force of viscous blood flow over the endothelium. Shear stress acts as the positive stimulus for endothelial cells and determines the gene expression pattern in these cells. Since force is a product of mass and acceleration, an increase in volume and/or velocity of blood flow increases positive stimulus to the endothelial cells. Shear stress affects gene expression in endothelial cells through trans-activating factors that bind to specific shear stress response elements (SSRE) in the promoters of shear stress responsive genes. The canonical SSRE sequence is GAGACC (Resnick et al., 1993). Shear stress activates a number of transcription factors, including KLF2 (Huddleson, Srinivasan, Ahmad, & Lingrel, 2004), which induces other endothelial specific genes like eNOS.

In the chicken embryo, KLF2 is expressed in the inner curvature of the heart and in high fluid shear force regions such as the outflow tract (OFT) and atrioventricular canal (AVC) region. A venous clip model was generated to study the effects of an altered blood flow pattern on cardiac development and gene expression. In this model, the right lateral vitelline vein of a chicken embryo was surgically ligated. In WT chicken embryos of stage HH18, KLF2 expression is restricted predominantly in the entire AV canal endocardial cell lining, in a few cells at the top of the ventricular trabeculations, and in the OFT. In the venous clip model, KLF2 was not expressed in the entire AV region, but only in the narrow region between the atrium and the ventricle. However in the rest of the heart, KLF2 expression was more widespread in the inner
lining of the atria and the ventricles as well as within the ventricular trabeculae (Groenendijk et al., 2004). This suggests that disrupting the blood flowing in to the heart, causes fluid shear stress and abnormally changes KLF2 expression pattern in the developing chicken heart endocardium.

Using the EOMA (mouse microvascular endothelial) cell line, it was shown that KLF2 expression increases within 24 hours of exposure to fluid shear forces in vitro (Huddleson et al., 2004, Hyde et al., 2012). In the mouse embryonic heart, an increase in KLF2 expression is correlated to the increase in blood flow from E8.5 to E14.5 and is localized to areas with high shear stress, like the OFT and AVC (Lee et al., 2006).

Huddleson et al. (Huddleson et al., 2004) used luciferase reporter assays to show that shear stress-induced transcriptional activation of KLF2 requires sequences from -157 to -95 bp (critical region) upstream of the transcriptional start site. In 2005, the same group used mobility shift assays and ChIP (Chromatin Immuno-precipitation) assays to show that the P-300 cAMP response element-binding protein-associated factor (PCAF) and heterogeneous nuclear ribonucleoprotein-D (hnrp D) bind the critical promoter region as components of a shear stress response cascade that is PI3K dependent. This pathway functions in acetylation of histones to increase KLF2 expression. Thus these results suggest a model where, in response to fluid shear forces, the PI3K signaling pathway is activated, leading to acetylation of histones surrounding the KLF2 promoter through PCAF acetyltransferase (Huddleson, Ahmad, Srinivasan, & Lingrel, 2005).

The role of KLF2 in the adult endothelium is to regulate the vascular barrier function. The endothelial barrier plays an important role in selective fluid and solute exchange. In inflammation induced adult KLF2+/- mice, leakage of Evan’s blue dye was observed, indicating
lack of barrier in major blood vessels. The study further showed that KLF2 positively regulates gene and consequently protein expression of the tight junction protein occludin in the endothelial cells. KLF2 also inhibits phosphorylation of the myosin light chain, and indirectly regulates adherens junctions as well (Lin et al., 2010). Oxidative stress or abundance of reactive oxygen species in the endothelial cells is known to cause adult cardiac abnormalities like hypoxia, ischemia, stroke and heart attacks. Oxidation of LDL causes plaque formation and arterial blockage. There is a study that correlates oxidative stress in pregnant females with congenital heart defects in the infants; however no specific defect was described (Hobbs et al., 2005). KLF2 protects adult endothelial cells under oxidative stress by enhancing the antioxidant activity of Nrf2 protein (Fledderus et al., 2008). Nrf2 is a transcription factor that positively regulates expression of antioxidant enzymes like heme oxygenase1 and NAD(P)H dehydrogenase quinone 1. If oxidative stress does affect embryonic cardiovascular development, KLF2 might activate protective antioxidant activity in embryos too.

In addition to its role in the cardiovascular system, KLF2 is important in the lungs, T cells, adipocytes and monocytes. The original name for KLF2 was LKLF, which stands for Lung Krüppel-like Factor (Kaczynski, Cook, & Urrutia, 2003; Wani, Means, & Lingrel, 1998). Experiments involving chimeric mice have shown that KLF2 plays a role in lung development (Wani, Means, & Lingrel, 1998) and also T cell differentiation (Kuo, Veselits, & Leiden, 1997). KLF2 is expressed in mature T and B cells in the quiescent state, and is downregulated when these cells are activated. Thus in the absence of KLF2, T cells stay in an activated state and are prone to apoptosis (Buckley, Kuo, & Leiden, 2001; Kuo, Veselits, & Leiden, 1997; Yusuf & Fruman, 2003). KLF2 also regulates migration of T cells from the thymus, thus controlling the circulation of peripheral T cells. It also controls differential trafficking of resting and activated T cells by regulating expression of the mediating chemokine receptors (Carlson et al., 2006; Sebzda, Zou, Lee, Wang, & Kahn, 2008). KLF2 is expressed in monocytes, and knockdown of
KLF2 leads to monocyte activation and subsequently, inflammatory gene expression (Das et al., 2006). A recent study showed that KLF2 plays an important role in regulating functional reactivity and subset differentiation of B-cells (Hart, Wang, Hogquist, & Jameson, 2011). KLF2 is expressed in adult adipocytes. It acts as an inhibitor of adipogenesis by repressing regulatory transcription factor Ppar\(\gamma\). KLF2\(-/-\) cell lines have shown to promote adipocyte differentiation (Banerjee et al., 2003; Wu, Srinivasan, Neumann, & Lingrel, 2005)

**Mouse heart development**

Because the heart is the main organ designated for the efficient, lifelong supply of oxygen to the whole body, along with the blood and the vascular system, it is important for it to start functioning early and keep adapting to its changing morphology during development. Thus the structural and functional development of the heart is interconnected. During the very initial stage of pumping action, the embryonic heart is a pulsating blood vessel with endocardial, extracellular matrix and myocardial layers. The pumping action of this tube differs considerably from the adult heart because it has to generate hemodynamically effective unidirectional blood flow in the absence of valves. Visual observations of the pulsating heart tube indicate that the pumping action is characterized by the cyclic generation of traveling mechanical waves from the venous to the arterial end. Fluid tends to flow from a region of higher to lower pressure.

*Heart tube formation*

The heart is the first organ to develop and function. It is required to support the dynamically growing embryo. Mouse heart development begins at E7.5, when cardiomyocytes differentiate from precursor cells and move anterior-laterally to form bilateral cardiogenic plates.
Endothelial cells differentiate subjacent to the cardiogenic plates and form right and left heart tubes (Kaufman MH & Bard JBL, 1999). The two cardiogenic fields fuse at the ventral midline and form a single beating heart with right and left sinus horns by E8.0. A regular heartbeat is established by E9.0. The left sinus acts as the initial pacemaker, and the wave of contraction is thus propagated along the tubular heart (Kaufman & Navaratnam, 1981). The myocardium forms the exterior of the heart tube and endothelial cells or endocardium forms the innermost layer. The space between the two layers consists of extracellular matrix namely cardiac jelly that is a product of localized secretion by the myocardium (Fig. 1-3). The heart tube loops to the right between E8.5 and 10.5. Towards the end of E8.5, the heart tube lengthens and ballooning occurs to form the chambers. The primitive chambers can be seen as the bulbus cordis (future right ventricle), primitive left ventricle, and common atrial chamber behind the primitive left ventricle. Myocardium from the anterior heart field helps to lengthen the outflow tract (OFT) after the formation of the heart tube (first heart field) (Kirby, 2002; Yelbuz et al., 2002). Beginning during E9.5, the right and the left sinus horns empty all of the blood in the common atrial chamber, which is connected to the primitive left ventricle with by an atrioventricular canal (AVC). The OFT connects the future right ventricle to the aortic sac. In the OFT and the AVC, localized swellings, termed endocardial cushions, arise at the inner endocardial layer (Kisanuki et al., 2001; Mjaatvedt & Markwald, 1989). These cushions are the primordia of the valvular and septal structure of the heart (Fananapazir & Kaufman, 1988). In mice, beginning at E10.5 the common atrial chamber divides to form right and left atria, and by E11.5 functional left and right ventricles are formed. The valves and septation are still in development at this stage (Nakajima, Yamagishi, Hokari, & Nakamura, 2000a)
The formation of the cardiac cushions requires a complex series of events characterized by endothelial-mesenchymal transformation (EMT) of the endothelial cells lining the OFT and AVC. By E9.0, the localized production of the cardiac jelly increases at the junction between the primordial atrium and left ventricle and in the outflow tract. Pulse chase radioactive labelling showed that 95% of the cardiac jelly in the cushions is secreted by the myocardium (Krug, Runyan, & Markwald, 1985a). The major components of the cardiac jelly are glycosaminoglycans, chondroitin sulphate and hyaluranon. These hydrophilic molecules promote swelling of the acellular space, making space for cushion formation (Manasek, 1975; Markwald & Smith, 1972; Markwald, Fitzharris, & Manasek, 1977). EMT occurs at the sites of these localized swellings. Turning an endothelial cell into a mesenchymal cell requires alterations in morphology, cellular architecture, adhesion and migration capacity. The biologic events that occur during EMT are delamination or loss of intercellular connections between the endothelial cells; endothelial cell hypertrophy or activation, and transformation, i.e., acquisition of cell migratory and invasive capacity (Fig. 1-4). Endothelial cells receive myocardial signals before they delaminate and swell. The swollen cells are closely packed and look like a cobblestone path, whereas delaminated cells reveal regions of cell-cell separation, as observed by scanning electron microscopy (Markwald, Fitzharris, & Smith, 1975). After separation, the transforming endocardial cells form numerous filopodia which assist in invasion and migration away from the endocardium into the cardiac jelly. The experimental system commonly used to demonstrate EMT is an ex vivo assay. The AV canal endocardial cushions are explanted onto hydrated type I collagen gels along with the myocardial layer. The presence of normal EMT is indicated by rapid separation and activation of endocardial cells and eventual transformation into star-shaped mesenchymal cells that are capable of migrating into the collagen gel (Bernanke & Markwald, 1982; Camenisch et al., 2000).
Remodelling and Chamber Septation

After AV endocardial cushion formation, extensive remodeling of the cardiac cushion takes place during valvulogenesis and septation. By E10.5 in mice, two different AV endocardial cushions are apparent – the ventral superior and the dorsal inferior endocardial cushion. The two cushions juxtapose and form a central cushion tissue called septum inter-medium or AV septum that forms the basic structure of the 4 chambered heart (Person, Klewer, & Runyan, 2005). During atrial septation, a membranous structure, the septum primum, appears on the posterior-superior aspect of the common primitive atrium medial to the entrance of the common venous sinus. The septum primum grows caudally and anteriorly until it meets the outgrowth of the central AV cushions. The ventricular septum is normally formed later in the embryo and depends on the fusion of 5 different components: the primitive ventricular septum, the posterior and the anterior AV cushions, and the dextro-dorsal and the sinistro-ventral conal ridges. Extensive remodeling of the AV endocardial cushions results in the bicuspid and tricuspid valves, whereas that of the outflow tract endocardial cushions results in the pulmonary valves of the adult heart.

Trabeculation

Trabeculae start forming in the heart at the heart tube stage, where they are often just ridge-like incursions of endocardium, running circumferentially. These are known as primary trabeculae. Forces produced by the heart beating stimulate patterned trabecular morphogenesis. Once the heart loops, there is lengthening of the primary trabeculae and emergence of offshoots, known as secondary trabeculae. Trabecular patterning has been experimentally shown to respond to hemodynamic forces. Myocardial trabeculae play an
important role in enhancing cardiac contractility, direct blood flow before septation, ventricular septation and intra-ventricular conduction.

**Apoptosis**

One of the common events during heart development is apoptosis, which occurs at specific times and regions and is an important part of remodeling. The primary sites of apoptosis are the OFT and AVC cushions and heart wall during development of the aortic and pulmonary valves and the interventricular septum. Apoptosis in the developing mouse heart begins around E14.5 and continues till right before birth at E20.5 (Icardo, 1996; Pexieder, 1975; Poelmann, Molin, Wisse, & Gittenberger-de Groot, 2000).
Figure 1-3: Schematic representation of cardiac development. Cardiogenic precursors form a crescent (left-most panel) that is specified to form specific segments of the linear heart tube, which is patterned along the anterior–posterior axis to form the various regions and chambers of the looped and mature heart. Each cardiac chamber balloons out from the outer curvature of the looped heart tube in a segmental fashion. Neural crest cells populate the bilaterally symmetrical aortic arch arteries (III, IV and VI) and aortic sac (AS) that together contribute to specific segments of the mature aortic arch, also colour coded. Mesenchymal cells form the cardiac valves from the conotruncal (CT) and atrioventricular valve (AVV) segments. Corresponding days of human embryonic development are indicated. A, atrium; Ao, aorta; DA, ductus arteriosus; LA, left atrium; LCC, left common carotid; LSCA, left subclavian artery; LV, left ventricle; PA, pulmonary artery; RA, right atrium; RCC, right common carotid; RSCA, right subclavian artery; RV, right ventricle; V, ventricle (28 Olson, E.N. 2006).
Figure 1-4: Anatomic overview of heart valve development. The developing heart tube contains an outer layer of myocardium and an inner lining of endothelial cells separated by an ECM referred to as the cardiac jelly. During heart valve formation, a subset of endothelial cells overlying the future valve site are specified to delaminate, differentiate, and migrate into the cardiac jelly, a process referred to as endothelial-mesenchymal transformation or transdifferentiation (EMT). Locally expanded swellings of cardiac jelly and mesenchymal cells are referred to as cardiac cushions. In a poorly understood process, cardiac cushions undergo extensive remodeling from bulbous swellings to eventual thinly tapered heart valves (99 Armstrong, E.J. 2004).
KLF2 in cardiovascular development

KLF2 has been extensively studied in vascular endothelial biology. The gene is expressed in the developing mouse embryo from E8.5 onwards. Absence of KLF2 is lethal and mouse embryos die between E12.5 and E14.5 (Kuo, Veselits et al., 1997; Lee et al., 2006; Wani et al., 1998). The specific cause of death and the specific mechanism of action in KLF2 knockout embryos are not unanimously agreed upon. Kuo et al. examined KLF2−/− embryos, and concluded that death is due to hemorrhaging and a lack of integrity in the tunica media of the blood vessels, including aortae and umbilical veins and arteries (Kuo, Veselits et al., 1997). Hemorrhaging in KLF2−/− embryos was also reported by another group (Wani et al., 1998). In another study, Wu et al., showed that KLF2−/− embryos have normal endothelial cell development, but a failure of mural cells to migrate around endothelial cells to stabilize the blood vessels (Wu, Bohanan, Neumann, & Lingrel, 2008). Platelet-derived growth factor (PDGF) is known to regulate VSMC migration. Using KLF2−/− mouse embryonic fibroblasts in culture, it was shown that KLF2 plays a role in PDGF-B induced migration. However none of these studies indicated the role of KLF2 in heart development.

The role of KLF2 in cardiac development was studied by Lee et al., in knockout (KO) mice in a mixed genetic background including C57BL/6J. The study involved in situ hybridization to detect expression of KLF2 in the developing heart as early as E8.5, in areas that have high fluid shear force. In KLF2 KO and KLF2 endothelial conditional KO embryos, a cardiac phenotype is observed as early as E10.5. This study did not report any hemorrhaging, or vascular smooth muscle cell (VSMC) abnormalities in the blood vessels. However the KLF2−/− embryos died from high output cardiac failure due to reduced smooth muscle tone. It was also noted that at E11.5, KLF2 endothelial conditional KO embryos had thinner myocardium
compared to the WT. This study supports the fact that endothelial expression of KLF2 is required for VSMC function in nascent vessels but eventually affects the heart development as a functional outcome. There was a zebrafish component to this study, wherein klf2a was knocked down to observe functional signs of heart failure and was rescued by the VSM stimulant phenylephrine (Lee et al., 2005.)

The zebrafish gene, klf2a, a homolog of KLF2 in mouse and man, is important in valve development as shown by another study. Knockdown of klf2a causes thicker and less flexible valves and increased regurgitation in the developing heart, compared to WT (Vermot et al., 2009). Valve development in zebrafish is divergent from that in mice. In mice, EMT produces mesenchymal cells in the cushion; in zebrafish, valves emerge directly through invagination of the AV endothelium. Invagination depends on reversing blood flow and the absence of it correlates with lack of endothelial cell shape change. One of the limitations of the research described above is that they have used a klf2a knockdown model. Unlike knockout, knockdown of a gene does not result in complete absence of the gene. This study supports a role of klf2a in valve development; however it may not completely correlate with the mechanism in mammals due to differences in zebrafish and mammalian heart structure and development.

Preliminary studies performed in our lab showed a novel and more severe effect of KLF2 knockout on cardiac development at E9.5. The animals studied were in an FVB/N genetic background. Serial sections of entire embryos were collected to assess morphological abnormalities. Using light microscopy, anterior to posterior cross-sections of E9.5 FVB/N KLF2-/- embryos were analyzed. Compared to WT littermates, these KLF2-/- embryos appear grossly normal, but at the cellular level there is a dramatic difference in the AV endocardial cushions. In WT embryos, the AV cushions are lined by a single layer of endothelial cells, as expected (Fig.
1-5A and 1-5C, n = 4). However in the FVB/N KLF2-/− embryos, there is an increased number of cells lining the AV canal region, and these cells form multiple disorganized layers (Fig. 1-5B and 1-5D, n = 4). The AV cushion region defects in E9.5 FVB/N KLF2-/− mice were studied at the subcellular level using transmission electron microscopy (TEM). TEM reveals that the E9.5 FVB/N WT (Fig. 1-6A) and the KLF2-/− (Fig. 1-6B) AV canals are patent and contain erythroid cells. The KLF2-/− AV canal is not lined by typical endocardial cells that are squamous (flat) but instead by cells that are bulbous with numerous cytoplasmic processes extending into the lumen of the AV canal (Fig. 1-6D). WT cells are squamous and either lack projections, or have them towards the cushions, rather than lumen, as expected (Fig. 1-6C). Moreover, additional cells lie adjacent to the endocardial layer in KLF2-/− compared to WT, which makes the cushions appear disorganized and non-laminar.
Figure 1-5: E9.5 FVB/N KLF2-/- atrioventricular cushions have accumulated cells lining the AV canal. (A) and (C) are WT (n = 4) and (B) and (D) are KLF2-/- (n= 4) hearts. Micrographs A and B (magnification 50X) show atrial (At) and ventricular (V) chambers. The boxes or brackets enclose the endocardial cushions (EC) shown at higher magnification in C and D (400X). Endo: Endocardial cells; Myo: Myocardium; Ery: erythroid cells; Mes: Mesenchymal cells. Experiments performed by Tina Lung.
Figure 1-6: Electron microscopy of E9.5 FVB/N KLF2/- atrioventricular cushions shows abnormal endocardial cell morphology. In WT (A), the endocardium is one cell-layer thick but in KLF2/- (B), there are multiple disorganized cell layers. The boxes enclose the AV canal. At higher magnification, endocardial cells in KLF2 KO (D) extend cytoplasmic projections or filopodia-like extensions towards the lumen of the AV canal, which are not observed in WT (C). Arrowhead indicates abnormal cytoplasmic projection towards lumen in KLF2-/. * indicates non-laminar cells accumulated at the lining of the AV canal that are observed in KLF2/- but not in WT. Electron microscopy was performed by Dr. Jack Haar and Tina Lung.
Molecular insights into cardiac and early valve development

A number of genes and associated signaling pathways have been found to be important in mouse cardiac development and endocardial cushion development. The majority of these genes are transcription factors, while others include growth factor genes and genes important for extracellular matrix synthesis.

**Transcription Factors in cardiac development**

Nuclear factor of activated T cells (NFATc) is expressed in the cardiac endocardium from E7.5 (de la Pompa et al., 1998). NFATc knockout mice show hypoplastic semilunar and AV valves and a ventricular septal defect from E12.5 and die by E14.5. NFATc expression is lost as endocardial cells transform into mesenchymal cells, making it a marker for absence of EMT (Ranger et al., 1998). NFATc is critical for endocardial cell proliferation but is also involved in remodelling of the valves and ventricular septum post EMT (Fig. 1-8) (Zhou et al., 2005).

Sox9 is a cardiovascular transcription factor expressed in endothelial and mesenchymal cells in the endocardial cushion region (Lincoln, Kist, Scherer, & Yutzey, 2007). Sox9 KO results in hypoplastic endocardial cushions and abnormal valve formation. These embryos die from cardiac insufficiency at E12.5 (Akiyama et al., 2004). A study by Lincoln et al. showed that ablation of Sox9 specifically in endocardial cells results in reduced EMT in mouse embryos. The reduction in EMT is attributed partially to reduced endocardial proliferation (Lincoln et al., 2007). During EMT in the absence of Sox9, NFATc is ectopically expressed in mesenchymal cells, indicating that transformation is aberrant (Akiyama et al., 2004). Thus the role of Sox9 in EMT is to promote the mesenchymal cell phenotype by inhibition of NFATc transcription. Sox9
positively regulates expression of the epidermal growth factor ErbB3, whose ablation shows a cardiac phenotype similar to Sox9-/-(Akiyama et al., 2004).

T-box 5 protein (Tbx5) is a transcription factor required for cardiac cushion and septum formation. Tbx-5 mutations cause Holt-Oram syndrome in humans, an autosomal dominant disorder characterized by skeletal and cardiac defects. Tbx5 is expressed in endocardial cells from E9.0 onwards (Basson et al., 1997).

The GATA family of transcription factors play an important role during cardiac development (Fig. 1-8). An endocardial specific Gata4 KO has multiple layers of endocardium in the AV canal, and hypocellular AV cushions at E10.5 (Rivera-Feliciano et al., 2006). Interestingly, the Tbx5 and Gata4 proteins physically interact during cardiac development. A heterozygous mutation (mG295S) in the Gata4 gene disrupts these protein interactions, resulting in cardiac defects like atrial septal defects (ASD), AV septal defects (AVSD) and myocardial thinning beginning at E11.5 (Garg et al., 2003; Maitra et al., 2009). The AVSD and ASD in these mice are known to result from abnormal EMT and remodeling of endocardial cushions. Gata4 mG295S is a missense mutation resulting in diminished DNA binding affinity and transcriptional activity, making it similar to a null allele. In mice with this Gata4 mutation and a null allele for Tbx5, Gata4+/−Tbx5+/−, there is normal EMT but defective remodeling, resulting in septal defects (Maitra et al., 2009).

Gata4 also interacts with FOG2 (Friend of Gata 2), resulting in synergistic activation or repression of Gata dependent cardiac promoters. FOG2 is expressed in developing and adult myocardium beginning at E9.5 (Flagg, Earley, & Svensson, 2007; Lu et al., 1999). Fog2−/−embryos die due to cardiac morphogenetic defects like thinner myocardium, and common atrioventricular canal i.e. AVSD and tetralogy of Fallot (Tevosian et al., 2000).
Nkx2.5 is another transcription factor expressed in the developing myocardium and is involved in early myocardial lineage specification. Nkx2.5 knockout mice have defects ranging from thinner myocardium to defective cardiac looping. Nkx2.5 is also reported to interact with Gata4 and Tbx5, and this interaction plays an important role in septal formation. In the absence of Nkx2.5, the position of the atrial septum is changed. This suggests that the myocardium also plays an important role in atrial septation.

**Cardiac genes important in cardiac jelly synthesis:**

The cardiac jelly components, including proteoglycans, glycosaminoglycans (hyaluronan and chondroitin sulphate), glycoproteins (fibronectin, laminin, fibulin, thrombospondin, cytoactin, vitronectin), collagens (I, III, IV) and other structural proteins, provide the framework for the developing heart (Markwald et al., 1978; Markwald, Fitzharris, Bank, & Bernanke, 1978; Nakamura & Manasek, 1981; Nakamura & Manasek, 1981).

The cardiac jelly underlying the endocardial cushion is comprised primarily of the glycosaminoglycan (GAG), hyaluronan (HA). UDP-Glucose dehydrogenase (UGDH) and Hyaluronan synthase (Has) synthesize HA at the plasma membrane and it is directly released from the myocardial cells. HA is an important cardiac jelly component in valvulogenesis (Hascall, 2000). Hyaluronidase treatments on hearts allowed endocardial activation but not migration during cushion EMT (Krug, Runyan, & Markwald, 1985a)(Krug, Runyan, & Markwald, 1985b). Mice null for Has2 do not form HA and completely lack cardiac cushion. Cardiac cushion endocardial and mesenchymal cells express Has2 during EMT. AV canal explants fail to undergo EMT in Has2-/- embryos. However, addition of exogenous HA reverts the phenotype.
(Camenisch et al., 2000). HA is thus required for EMT; however, whether its role is direct or indirect is not known.

UDP-Glucose Dehydrogenase is an enzyme required for the conversion of UDP-Glucose to UDP-Glucuronic acid, which is further used in the biosynthesis of HA, GAGs, heparin sulphate and chondroitin (Fig 1-7). Zebrafish heterozygous for Jekyll, a gene encoding UGDH, fail to develop atrioventricular valves (Hyde et al., 2012).

Figure 1-7: Network of cardiac jelly components. Flow chart resembles synthesis of cardiac jelly components. The enzymes involved are represented in orange, the cardiac jelly molecules in blue and the signaling pathway that these molecules lead to is green. UDPG: UDP-Glucose; UGDH: UDP-Glucose dehydrogenase; UDPGA: UDP – Glucuronic acid; Has2: Hyaluronan synthase 2.
Other cardiac genes and signaling pathway molecules in EMT

A number of molecules and signaling pathways, other than transcription factors, are known to play an important role in EMT during endocardial cushion development. Notch1 is expressed in endocardial cells from E8.0 and is known to play a pivotal role in cardiac cushion EMT (Table 1-1; Fig. 1-8) (Timmerman et al., 2004). Notch1−/− embryos die by embryonic day E10.5 due to a number of cardiac and vascular abnormalities (High & Epstein, 2008). Notch1−/− embryos have hypocellular cardiac cushions, with respect to mesenchymal cells, and reduced trabeculation of the ventricular wall. Hesr1 and Hesr2 are targets of the Notch1 signaling pathway. Double knock-out (KO) embryos for Hesr1 and Hesr2 show a similar cardiac phenotype as Notch1−/− (Kokubo, Tomita-Miyagawa, Hamada, & Saga, 2007a).

Bone morphogenetic protein 2 (BMP2) is expressed in the myocardium from E8.0 until endocardial cushions begin to form at E10.5, and then it is expressed in cushion mesenchymal cells (Table 1-1; Fig. 1-8) (Sugi, Yamamura, Okagawa, & Markwald, 2004b). BMP2−/− mice die by E8.5; however an AV myocardium conditional KO of BMP2 showed that BMP2−/− cardiac cells are incapable of EMT induction and cardiac jelly accumulation (Ma, Lu, Schwartz, & Martin, 2005a) . Thus BMP2 signaling also plays an important role in cardiac EMT via myocardial signaling, even though BMP2 is not expressed in endocardial cells. Msx1 and Msx2 are closely related downstream effectors of BMP signaling, and are expressed in the heart endocardium and myocardium from E9.5. Deficiency of Msx1 and Msx2 in mouse embryos results in hypoplastic AV cushions, with respect to mesenchymal cells, by E10.5 (Chen, Ishii, Sucov, & Maxson, 2008a). Thus Msx1/2 form a link between myocardial and endocardial signaling during AV cushion development. Double KO Msx1 and Msx2 embryos show reduced expression of mesenchymal hyaluronan synthase 2 (Has2) (Camenisch et al., 2000).
Hyaluronan activates ErbB2-ErbB3 receptors that are responsible for heart valve mesenchyme formation. Camenisch et al (2002) showed that in Has2-/- embryos, restoration of ErbB2 and ErbB3 phosphorylation or heregulin treatment results in normal EMT and formation of mesenchymal cells (Camenisch, Schroeder, Bradley, Klewer, & McDonald, 2002).

Endoglin is a transmembrane accessory receptor for a number of signaling pathways including those involving the TGF-beta superfamily and BMP2. It is expressed in endocardial cells. Eng-/- embryos die by E10.5 due to circulatory arrest and hemorrhaging. However these embryos show reduced EMT resulting in hypoplastic endocardial cushions at 10.5 (Table 1-1) (Nomura-Kitabayashi, Anderson, Sleep, Mena, Karabegovic, Karamath, Letarte, & Puri, 2009b).

The above mentioned and a few additional cardiovascular genes and their expression and functional details are listed in Table 1-1. Figure 1-8 summarizes the molecular regulation of the AV endocardial cushion development. It also illustrates the specific location of expression and function of these molecules and their interaction with each other.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Expressed from embryonic day and location of expression</th>
<th>KO phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bmp2</td>
<td>E9.25 Myo</td>
<td>Bmp2 knockout results in absence of cardiac jelly and consequently AV canal EMT.</td>
<td>(Ma, Lu, Schwartz, &amp; Martin, 2005a)</td>
</tr>
<tr>
<td>Msx1 and Msx2</td>
<td>E9.5 Endo, Myo</td>
<td>Absence of EMT. Hypocellular endocardial cushions.</td>
<td>(Chen, Ishii, Sucov, &amp; Maxson, 2008a)</td>
</tr>
<tr>
<td>Sox9</td>
<td>E9.5 Endo, Mes</td>
<td>Endocardial cell migration is absent. Immature extracellular matrix.</td>
<td>(Akiyama et al., 2004; Lincoln et al., 2007)</td>
</tr>
<tr>
<td>Has2</td>
<td>E8.5 Endo, Myo, Mes</td>
<td>No endocardial cushion swelling. Alcian blue negative.</td>
<td>(Camenisch et al., 2000)</td>
</tr>
<tr>
<td>Hey1/Hesr 1</td>
<td>E9.0 onwards Endo, Myo</td>
<td>Reduced ventricular wall trabeculation, hypocellular AV cushions absence of EMT.</td>
<td>(Kokubo, Tomita-Miyagawa, Hamada, &amp; Saga, 2007a)</td>
</tr>
<tr>
<td>Gata4</td>
<td>E9.0 Endo, Myo</td>
<td>Endocardial cushion, Reduced ventricular wall trabeculation, VSD and ASD.</td>
<td>(Kuo et al., 1997)</td>
</tr>
<tr>
<td>Tbx5</td>
<td>E9.0 onwards Endo</td>
<td>Hypoplastic endocardial cushion and ASD.</td>
<td>(Basson et al., 1997)</td>
</tr>
<tr>
<td>Notch1</td>
<td>E9.5 day Endo</td>
<td>Hypocellular endocardial cushions</td>
<td>(Timmerman et al., 2004)</td>
</tr>
<tr>
<td>Tgfβ2</td>
<td>E8.0 onwards Endo, Myo</td>
<td>Hypoplastic AV cushion development, myocardial defect</td>
<td></td>
</tr>
<tr>
<td>UGDH</td>
<td>E6.5 onwards Endo</td>
<td>No glycosaminoglycan synthesis. KO die by E8.0</td>
<td>(Hyde et al., 2012)</td>
</tr>
<tr>
<td>Endoglin</td>
<td>E8.0 Endo</td>
<td>Hypocellular AV and OFT cushions</td>
<td>(Nomura-Kitabayashi, Anderson, Sleep, Mena, Karabegovic, Karamath, Letarte, &amp; Puri, 2009a)</td>
</tr>
<tr>
<td>ErbB3</td>
<td>Endo, Mes</td>
<td>Hypocellular AV and OFT cushions</td>
<td>(Camenisch et al., 2002)</td>
</tr>
<tr>
<td>Alk3</td>
<td>Endo, Myo, Mes</td>
<td>Myo inactivation, AV and septum defects</td>
<td>(Smith et al., 2009)</td>
</tr>
<tr>
<td>Periostin</td>
<td>Endo E9.5</td>
<td>Post-EMT remodelling</td>
<td></td>
</tr>
<tr>
<td>Fog2</td>
<td>E9.5 onwards Endo</td>
<td>Attenuates EMT and septal defects</td>
<td>(Flagg et al., 2007)</td>
</tr>
</tbody>
</table>

Endo – endocardium; Myo – Myocardium; Mes – Mesenchymal cells
Figure 1-8: Molecules that regulate endocardial cushion EMT. Endocardial EMT is regulated by signaling between atrioventricular canal (AVC) myocardium and overlying cushion endocardium. Cardiac jelly, the extracellular matrix of the cushions, is also required for endocardial EMT. (Reviewed in (155 von Gise, A. 2012)). Illustration credit: Cosmocyte/Ben Smith).
Clinical Significance

Heart development requires a combination of complex morphogenetic events and hemodynamic forces, thus making it vulnerable to molecular abnormalities. Congenital heart defects (CHDs) affect 4 to 8 infants per 1000 births, and are a leading cause of infant morbidity and mortality (Reviewed in (Kirby, 2002)). Of all the CHDs, valve and septal defects account for the majority of the defects. CHDs can be caused by single gene mutations or their cause can be multifactorial. Despite the ongoing research in the field, very few CHD-causing genes have been identified. Genetic and molecular analysis using online databases and software programs have identified a few cardiac regulators and their functions. However the complete mechanism of action of very few of these regulators is known. A number of molecular pathways seem to play an important role during cardiac development. However there are missing links between them. There are a number of transcription factors that have been shown to be important in normal valve development in humans. An example is Nkx2-5, mutation in which causes diverse cardiac defects like atrial septal defect, ventricular non-compaction and AV block in adults that leads to syncope and sudden death (Reviewed in (Yelbuz et al., 2002)). Mutations in a cardiac T-box gene, TBX5, cause Holt-Oram syndrome. Life threatening cardiac abnormalities are observed in 75% of the individuals with Holt-Oram Syndrome. These mainly include atrial and septal defects and/ or cardiac conduction defects leading to bradycardia and fibrillation. The elucidation of mechanisms of Nkx2-5, Tbx5 and other transcription factors, like KLF2, will provide a molecular framework that will continue to further our understanding of normal heart development.

Our preliminary data indicates that at E9.5 and E10.5, KLF2-/- embryos have defects in the AV endocardial cushions that further develop into the AV valves and septum. Because KLF2 is a transcription factor, it has the capacity to regulate expression of other genes in
cardiovascular development. Thus it could play an important role in connecting various molecular pathways. KLF2 has never before been reported in the context of congenital flow defects. It is a novel player. This research thus has implications for understanding the biology of and thereby treating valve defects.

Implications of genetic background in cardiac development

The genetic background of mice affects the phenotypic outcome of a genotype. Each strain has unique background alleles that may interact with and modify the expression of a mutation, transgene, or other genetic insert. The likelihood of such modifier genes having a confounding effect is especially high in an uncharacterized background or in a segregating or mixed background of unspecified origin. Even in a well-characterized strain, undiscovered modifier genes may confound results, sometimes making them unexplainable. Such modifier genes are the reason why normal development and physiology often vary significantly among inbred strains. Inbred strains are homologous at all loci.

The importance of genetic background in cardiac development has been demonstrated by a number of studies. Rajagopal et al. demonstrated that the rate of survival of GATA4 delta mutant (G4D) mice in a C57 (G4D-C57) genetic background was decreased and that these mice had higher atrial septal defects (ASDs) and ventricular septal defects (VSDs) in neonates when compared to mice in an FVB background (G4D-FVB). They further crossed G4D-C57 to G4D-FVB mice and obtained G4D-F1 mice that had 50% of each background. They found that hearts of F1 mice were normal and a lower frequency of septal defects than G4D-C57. The WT survival rate of both the strains is the same. Thus, the above data suggests that genetic
modifiers for increased risk of septal defects are recessive in the C57BL/6 strain (Rajagopal et al., 2007).

Astrof et al showed that heart development in fibronectin null (FN-/-) mice leads to early embryonic lethality in both 129S4 and C57BL/6J strains. Heart looping is absent in 129S4 FN-/- embryos, which die by E9.0. However in C57BL/6J FN-/- embryos, heart looping does occur. To find the genetic modifiers involved, they performed genetic mapping and haplotype analyses. They used a genome-wide panel of SNP markers spaced about 20cM apart on each chromosome. These analyses led to linkage on chromosome 4. There were 21 genes in this 1Mbp region, of which, 5 genes were differentially expressed in the two strains and are potential modifiers (Astrof, Kirby, Lindblad-Toh, Daly, & Hynes, 2007).

**Rationale and Hypothesis**

KLF2 is expressed in the mouse endocardial cushion region, klf2a knockdown results in abnormal zebrafish heart valve development, and KLF2 knockout in mouse embryos results in abnormal AV cushion region development. This suggests that KLF2 may be important in the early stages of mammalian valve development. Previous work in the lab demonstrated that E9.5 KLF2-/- hearts in FVB/N genetic background are hyperplastic with respect to cells lining the AV canal, and hypoplastic with respect to endocardial cushion mesenchymal cells even at later stage of development than E9.5.

Thus the primary hypothesis of this study is that KLF2 plays an important role in cardiovascular development, primarily AV endocardial cushion development. Further, the effect of KLF2 knockout is dependent on the genetic background.
Based on the accumulation of cells lining the AV canal in E9.5 FVB/N embryos (Fig. 1-5), we hypothesize that KLF2-/− embryos have abnormal endothelial to mesenchymal transition (EMT). To examine EMT we performed descriptive morphological studies and explant endothelial cell migration assays. Cardiac jelly plays an important role in regulating EMT. Thus Alcian blue staining was performed to assess the presence of glycosaminoglycans in the cardiac jelly. To study how the molecular phenotype is translated into cardiac function, echocardiography was performed to compare cardiac function parameters in E10.5 WT and KLF2-/− embryos. KLF2, being a transcription factor, potentially regulates other genes important in cardiac development.

To study the molecular mechanism of KLF2 action, we performed qRT-PCR to study expression a set of genes selected on the basis of the literature. For the genes that were differentially expressed in KLF2-/− and WT AV regions, we performed chromatin Immunoprecipitation assays using an anti-KLF2 antibody to show that KLF2 binds the promoter regions of these genes.
Summary

Krüppel-like factor 2 (KLF2) is expressed in endothelial cells in the developing heart, particularly in areas of high shear stress, such as the atroventricular canal (AVC). KLF2 ablation leads to myocardial thinning, high output cardiac failure and death by mouse embryonic day 14.5 (E14.5) in a mixed genetic background. This chapter identifies an earlier and more fundamental role for KLF2 in mouse cardiac development in FVB/N mice. FVB/N KLF2-/- embryos die earlier, by E11.5. E9.5 FVB/N KLF2-/- hearts have multiple, disorganized cell layers lining the AV cushions, the primordia of the AV valves, rather than the normal single layer. This results in higher number of cells lining the AV canal and a reduced AV canal volume in mutant hearts compared to WT. By E10.5, traditional and endothelial-specific FVB/N KLF2-/- AV cushions are hypocellular with respect to mesenchymal cells, suggesting that the cells accumulating at the AV canal have a defect in endothelial to mesenchymal transformation (EMT). E10.5 FVB/N KLF2-/- hearts have reduced glycosaminoglycans in the cardiac jelly, correlating with the reduced EMT. However, the number of mesenchymal cells migrating from FVB/N KLF2-/- AV explants into a collagen matrix is reduced considerably compared to wild-type, suggesting that the EMT defect is not due solely to abnormal cardiac jelly. FVB/N KLF2-/- heart also shows delayed atrial septation compared to WT. In summary, cardiac defects in KLF2-/- mouse embryos are manifested differently in different genetic backgrounds.
Introduction

Congenital heart defects (CHDs) are a leading cause of infant morbidity and mortality (reviewed in (Pierpont et al., 2007)). Valve and septal defects account for the majority of CHDs. Mutations in certain transcription factor genes, including Nkx2-5 (Snyder, Huang, & Zhang, 2010) and the cardiac T-box gene TBX5 (Basson et al., 1997), are known to be important for normal valve development in humans. However, mutations of these genes account for only a small percentage of CHDs. Thus, mutations or variants in other transcription factor genes, like KLF2, are likely to be involved in valve defects.

During embryonic development heart forms as a primitive tube containing endocardial and myocardial cells separated by the extracellular matrix (ECM). The first indication of valve morphogenesis is localized swellings of the endocardial layer that arise at approximately embryonic day 9.5 (E9.5), and form the endocardial cushions of the atrioventricular (AV) canal and the outflow tract. The endocardial cushions are formed by endothelial to mesenchymal transformation (EMT). During EMT, AV endocardial cushion cells undergo hypertrophy, loss of cell-cell contacts, lateral mobility, formation of mesenchymal-like cell processes (filopodia), and migration into the cardiac jelly (Reviewed in (Lim & Thiery, 2012; Person et al., 2005)). This EMT generates mesenchymal progenitor cells that contribute to valvuloseptal structures and adult valve interstitial cells (VICs) (Griffin, Brennan, & Magnuson, 2008; Redmond, Dumur, Archer, Haar, & Lloyd, 2008). Without normal cardiac jelly, endothelial cells fail to transform and to migrate, resulting in hypoplastic endocardial cushions (Krug, Runyan, & Markwald, 1985a). Extensive remodeling and proliferation of the endocardial cushions occurs to form the adult heart valves. By E10.5, the right and left atria have divided (Nakajima, Yamagishi, Hokari, &...
Nakamura, 2000a); the AV endocardial cushion region also plays an important role in septation of the heart (Reviewed in (Person et al., 2005)).

**Table 2-1: Cellular composition of the heart during early development**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Origin</th>
<th>Location</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endocardium</td>
<td>From Flk-1 positive precardiac progenitors by de novo vasculogenesis during late primitive streak</td>
<td>Innermost lining of the heart from the heart tube stage</td>
<td>Form an endothelial layer within the heart that is contiguous with rest of the vasculature; Undergo EMT to form endocardial cushions that are primordial of the valves in the adult heart; Signaling with cardiomyocytes to form myocardial trabeculation and extracellular matrix; Signaling to facilitate transdifferentiation of myocytes to Purkinje fiber cells of the cardiac conduction system.</td>
</tr>
<tr>
<td>Myocardium</td>
<td>From Flk-1 positive precardiac progenitors by de novo vasculogenesis during late primitive streak</td>
<td>Outermost lining of the heart since the heart tube stage</td>
<td>Signaling to synthesize extracellular matrix, regulation of physiological function of the heart.</td>
</tr>
<tr>
<td>Mesenchymal cells</td>
<td>Mesenchymal cells of the endocardial cushions are derived from endocardial cells by EMT; Neural crest derived mesenchymal cells</td>
<td>Mesenchymal cells are major components of the AV and OFT endocardial cushions. Primordial atrial septum</td>
<td>Essential role in the formation of the AV endocardial cushions and AV septal components; Neural crest derived mesenchymal cells form OFT endocardial cushions.</td>
</tr>
<tr>
<td>Epicardium</td>
<td>Derived from a cluster of mesothelial cells developing at the base of the venous inflow tract of the early embryonic heart. This cell cluster is termed the proepicardium and gives rise not only to the epicardium but also to epicardium-derived cells.</td>
<td>Covers the entire heart and the intrapericardial region</td>
<td>Induce myocardial development and signaling with endocardium to facilitate trans-differentiation of myocytes to Purkinje fibers.</td>
</tr>
</tbody>
</table>
Krüppel-like factor 2 (KLF2) is a member of a family of zinc finger-containing transcription factors (Bieker, 2001; Kaczynski et al., 2003; McConnell & Yang, 2010). In the E9.5 mouse heart, KLF2 mRNA is highly expressed in the endocardial cells of the AV cushion regions (Lee et al., 2006), where it is likely induced by high shear stress. The role of KLF2 in cardiac development was studied by Lee et al., in knockout (KO) mice in a mixed genetic background including C57BL/6J. In KLF2 KO and KLF2 endothelial conditional KO embryos, a cardiac phenotype is observed as early as E10.5. These mice have thinning of the myocardium, high output heart failure, and die by E14.5. These KLF2-/- embryos also have cardiac functional defects due to loss of vessel tone, but no role for KLF2 in cushion formation was noted (Lee et al., 2006). In two other KLF2-/- models, embryos in a mixed genetic background die between E12.5 and E14.5, and exhibit hemorrhaging (Kuo, Veselits et al., 1997; Lee et al., 2006; Wani et al., 1998). The zebrafish gene, klf2a, a homolog of KLF2 in mouse and man, is important in valve development. Knockdown of klf2a causes thicker and less flexible valves and increased regurgitation in the developing heart, compared to WT (Vermot et al., 2009).

KLF2 is expressed in the mouse endocardial cushion region. This suggests that KLF2 may be important in the early stages of mammalian valve development. In this work, we show that FVB/N KLF2-/- hearts are hyperplastic with respect to cells lining the AV canal, and hypoplastic with respect to endocardial cushion mesenchymal cells. The data suggests that KLF2 regulates EMT, and also atrial septation. In summary, KLF2 activates multiple important cardiovascular development genes, suggesting mechanisms for its roles in the embryonic heart.
Methods

Generation of knockout and transgenic mice

The traditional KLF2 KO mouse model was developed by targeting the gene with the hypoxanthine phosphoribosyl-transferase (Hprt) gene [(Wani et al., 1998)]. KLF2+/− adults were mated with FVB/N or C57BL/6 mice for at least 12 generations to obtain KLF2+/− animals in the FVB/N or C57BL/6 genetic background. These animals were then mated to obtain KLF2−/− embryos in the respective genetic backgrounds. FVB/N KLF2+/− animals were mated with C57BL/6 KLF2+/+ mice to obtain KLF2+/− animals in a 50% FVB/N and 50% C57BL/6 genetic background (mixKLF2+/−). mixKLF2+/− adults were mated to obtain mixWT, mixKLF2+/− and mixKLF2−/− embryos and adult animals.

Tie2-cre transgenic animals were purchased from Jackson Laboratories (Bar Harbor, ME). Mice with a KLF2 allele flanked by loxP sites (floxed allele or KLF2fl/+)) were kindly provided by Dr. Jerry Lingrel, University of Cincinnati and were generated as previously described (Lingrel et al., 2012). The loxP sites in these mice are inserted such that they flank exon2 of KLF2, which encodes 271 of the total 354 amino acid residues. Cre recombinase-mediated excision at the loxP sites results in deletion of exon 2, with introduction of a frameshift in the downstream transcript. These animals were mated with FVB/N mice for at least 12 generations to obtain Tie2-cre and KLF2fl/fl animals in an FVB/N genetic background. FVB/N Tie2-cre and KLF2fl/fl animals were then mated with each other to obtain Tie2-cre, KLF2fl/+ mice, which were subsequently mated with KLF2fl/fl animals to obtain Tie2-cre, KLF2fl/fl animals in FVB/N background. These animals are designated FVB/N Tie2-cre KLF2−/−.
**Genotyping**

The offspring of KLF2+/- matings were screened for the normal and KLF2 knockout alleles by Polymerase Chain Reaction (PCR) using the following primers: Forward normal primer: 5'TTGTTTAGGTCTCATCCGTGCCG 3'; Reverse Normal Primer: 5' TTGCCGTCCTTTGCCACTTTGC 3'; Forward knockout primer: 5' TGCTTACACCTCCTAAATGT 3'; Reverse knockout primer: 5' CCTACCCGCTTCCATGCTC 3'. For genotyping, mouse ear clippings or embryo tail tissue were obtained and digested in 50µl of PCR digestion buffer (50mM KCl, 10mM Tris-HCl (pH8.5), 40mM MgCl2, 0.45% Nonidet P40 lysis buffer, 0.45% Tween 20) and 5µl of Proteinase K (10mg/ ml; Roche). The tubes were vortexed and incubated at 60°C overnight. Proteinase K is serine protease that cleaves the carboxylic ends of amino acids. The next day, samples were boiled twice for 10 minutes to inactivate the Proteinase K. Between the two boiling steps, the tubes were held at 4°C till cool to the touch and then zip-spun in an Eppendorf 5415C micro-centrifuge (Eppendorf, Westbury, NJ) for 5 seconds. The digested samples were stored at -20°C until ready for PCR. KLF2 PCR grand mix was prepared with 1X PCR buffer (without MgCl2), 250µM of each dNTP, 1.5mM MgCl2, 0.5µM of wild type and knock out primers. The grand mix is stored at – 20°C. For the PCR reaction, 6.75µl of the grand mix (per reaction), 0.2U/ µl of the Taq Polymerase, 0.175µg/ µl of RNAse A and molecular grade H2O was added to 1.5 ml microcentrifuge tube. One microliter of the DNA sample was added to 24µl of the above PCR mix in 0.2ml PCR tubes. Molecular grade H2O was used as a negative control and previously genotyped KLF2+/+, KLF2+/- and KLF2-/- samples were used as positive controls. All the samples and controls were run in duplicate to confirm the results. MJ Research PTC 100 thermocycler was used with following cycling conditions; 1 cycle 94°C for 3 minutes, 58°C for 1.5 minutes, 72°C for 1 minute 15 seconds, followed by 32 cycles at 94°C for 40 seconds, 58°C for 45 seconds and 72°C
C for minutes. The PCR products were analyzed on 2% agarose gels by electrophoresis in Tris acetate EDTA (TAE) buffer.

The presence of the Tie2-cre allele was screened similarly by PCR using following primers:

Forward Primer: 5’ GAATCCTGGATGCTAAGTTA 3’; Reverse Primer: 5’ AGTTCTGGACATGATGATA 3’.

The screening of the floxed allele of KLF2 was similarly performed using: Forward Primer 5’-GGAGGTAGACTCAGGCTGTG-3’ and reverse primer: 5’ GTTGTTTAGGTCTCCTCAGTG-3’. The presence or absence of the loxP site results in a 260-bp or 230-bp product size, respectively.

**Dissection and sample preparation**

KLF2+/- male and female mice in FVB/N, C57BL/6 and mixed genetic background were used in timed matings to generate KLF2+/+, KLF2+/- and KLF2-/- embryos. Animals were set up in mating and the females were checked for vaginal plugs (male semen coagulation) every morning. The presence of a vaginal plug indicated possible conception and establishing the time of gestation as 0.5 days post-coitus or embryonic day 0.5 (E0.5). On the 10th day following vaginal plug visualization (E10.5), the female was exposed to inhalation anesthesia (1ml Isoflurane) for 30 seconds in a closed 1L container and followed by injecting with 2.5% Avertin (10 g tribromoethanol, 10 mL tert-amyl alcohol) at approximately 0.015 mL/g body mass. A pinch to the lower limb indicated effective sedation. The pregnant female was then subjected to mechanical cervical dislocation. The uterine horns were removed and placed in 1X PBS to keep them hydrated. The embryos were dissected out of the maternal decidua and embryonic chorion using watchmaker’s forceps and surgical scissors in PBS, magnified (6.7X – 40.5X) with an Olympus SZ2-ILST dissection microscope. The yolk sac and embryo were detached and
photographed on a black background with an Olympus Q-Color 3 camera using QCapture 2.81.0 imaging software, after aligning the embryonic spine with a metric ruler. The developmental age was confirmed by somite counts (31–36 somites for E10.5; 29-31 somites for E9.5). The yolk sac tissue was used for genotyping and the embryo was stored in tool necked glass vials containing the fixative. For morphological analysis, the embryos were fixed in 2% paraformaldehyde in Millonig’s buffer (109mM NaOH and 136mM sodium biphosphate) and 0.25% glutaraldehyde. For immunohistochemistry, the embryos were fixed in 4% paraformaldehyde in Millonig’s buffer, without glutaraldehyde. Glutaraldehyde is an aggressive cross-linker, which is useful for maintaining tissue integrity during harsh procedures like plastic embedding. However it can mask antigens during cross-linking and hence hinder immunohistochemical reactions. The embryos were removed from the fixative after 24 hours, washed thrice and stored in Millonig’s buffer until ready for further use.

FVB/N Tie2-cre KLF2-/- embryos were dissected, fixed and stored as described above.

**Plastic Embedding, Sectioning and Staining for Light Microscopy**

For morphological analysis of the embryo using light microscopy, the embryo was osmicated at room temperature for 40 – 60 minutes in 1% Osmium tetroxide. The embryos were serially-dehydrated in 30%, 40%, 50%, 60%, and 70% ethanol for 5 minutes each and 80%, 95%, 100%, 100% ethanol for 10 minutes each. The specimens were transferred to a 1:1 mixture of ethanol and propylene oxide for 10 minutes, and 100% propylene oxide for 10 minutes, twice to remove residual ethanol. The samples were held overnight in a 1:1 mixture of propylene oxide and eponate 12 resin, and transferred the next day to 100% eponate 12 resin for several hours. The specimens were carefully removed with a toothpick and placed in a cutting-block mold with eponate 12 resin, and incubated in an oven at 55 °C for 2.5 days. Each embryo was placed
anterior to posterior along the length of the cylindrical mold. The plastic blocks were removed from their molds and trimmed to size.

Plastic embedded specimens were cut in 5-7µm thick cross-sections, using a Sorvall JB4 Microtome. Sections were carefully placed on Fisher Scientific Superfrost Plus microscopy slides, spotted with water to encourage section adhesion. Water was slowly vaporized on a hot plate before staining with a solution composed of 1% sodium borate, 1% azure II, 1% toluidine, and 1% methylene. Stained sections were photographed with an Olympus DP71 digital camera, mounted to an Olympus BX41 compound microscope, visualized with Olympus DP Controller 3.2.1.276 imaging software.

Cell counts and AV canal volume

Cells lining the AV canal of E9.5 embryos were counted in light micrographs taken at 200X magnification (10X eyepiece and 20X objective). The cells were counted from the central section among all the sections showing AV canal plus a section 2 sections anterior and a section 2 sections posterior to the central section. The ends of the AV canal were designated as 2 endothelial cells beyond the point where the AV canal widens into the ventricle or the atria. A rectangle of the length (in µm) of AV canal (described above) and width of 175µm was placed around the AV canal. All the cells lining the AV canal and accumulated in the AV region, except erythroid cells, were counted and compared between E9.5 WT and KLF2-/- hearts. The counts were expressed in cells/mm².

The volume of the E9.5 AV canal was determined in WT and KLF2-/- embryo hearts. For every embryo, all of the sections showing AV canal were selected. Image J 1.46 software was
used to trace the irregular outline of the AV canal and calculate the area of the canal. The area for each AV canal section was multiplied by 7 μm, to obtain the volume. The volumes for all sections were summed to obtain the total volume of the AV canal for each embryo. The ends of the AV canal were designated as 2 endothelial cells beyond the point where the AV canal widens into the ventricle or the atria. The volume was compared for WT and KLF2-/- hearts.

Endocardial cushion mesenchymal cells were counted for E10.5 WT and KLF2-/- embryos in all genetic backgrounds. The central section among all of the sections within the endocardial cushion was used to count star-shaped mesenchymal cells. Light micrographs at 200X magnification were used. The counts were expressed as cells/mm². The area of the cushion region was measured using Image J 1.46 software. Figure 2-1 shows an example of a WT endocardial cushion used to count the mesenchymal cells and measure the area.
Whole Mount Immunohistochemistry

KLF2−/− embryos were fixed overnight with 4% paraformaldehyde in PBS, washed with PBS four times, treated through serial ethanol dehydration: 30%, 50%, 70%, 95%, 100%, 100%, 100% and stored at -20°C. Samples were rehydrated stepwise to PBS with 0.1% Polysorbate 20 (PBST): 30%, 50%, 70%, 95%, 100%, and 100%. The embryos were washed four times in PBST for 15 min each, followed by incubation in 0.5% hydrogen peroxide, 0.5% serum in PBST for 30 min at room temperature. The samples were then placed in antibody blocking solution (10% goat serum in PBST) for 2 hr at room temperature, and were then incubated overnight at 4°C in primary antibody, PECAM (1:200) in antibody blocking solution. The next day, the embryos were washed four times for 30 min in antibody blocking solution. The embryos were

Figure 2-1: AV endocardial cushion mesenchymal cell count. The area within the red line is the AV endocardial cushion. This area was measured using Image J. Mesenchymal cells indicated by arrows within the endocardial cushion region were counted and the number of cells relative to the area were calculated.
then placed in secondary antibody, biotinylated Anti-Rat IgG (1:500) for 2 hr at room temperature. The samples were again washed in PBST as before, and incubated in pre-diluted BD Pharmingen Streptavidin-Horseradish Peroxidase (Sav-HRP) for 1 hr at room temperature. After washing four times for 30 min in PBST, the embryos were incubated in BD Pharmingen 3,3’diaminobenzidine (DAB) chromogen in H2O2 buffer for approximately 5 min. After washing with PBST to remove the DAB, the embryos were fixed overnight in 4% PFA in PBS at 4°C. The next day, the embryos were washed in PBS and cryo-embedded in OCT medium. They were then cut in 10µm thick sections using a vibratome (Ultrapro 5000). Coverslips were placed on the slides and digital pictures were taken with an Olympus DP71 digital camera mounted to an Olympus BX41 compound microscope, and visualized with Olympus DP Controller 3.2.1.276 imaging software.

**Alcian Blue staining**

E10.5 WT and KLF2-/− embryos were fixed in a 2% PFA and 0.25% glutaraldehyde solution. The embryos were then sequentially placed in 10%, 20%, 50% sucrose solutions for 1 hour each and left in 100% sucrose solution till the embryos sank to the bottom of the tube (overnight). Sucrose improves the tissue integrity during cryo-sectioning. Cross-sections of 10 µm were cut using a vibratome (Ultrapro 5000). Sections were stained with 1% Alcian Blue 8GX (Sigma Aldrich) in 3% Glacial Acetic acid (in distilled water) for 30 minutes. The pH of the Alcian Blue stain was maintained to 2.5. The slides were then washed under running tap water for 5 minutes and rinsed with distilled water to remove the residual stain completely. The sections were counter stained with 0.1% Nuclear Fast Red in 5% aluminum sulfate (Sigma Aldrich) for 5 minutes. Alcian blue stains the extracellular matrix blue and nuclear fast red stains the cells red, to create the contrast. The sections were washed in running tap water for 1 minute, air dried and
observed using an Olympus BX41 microscope. Images were made using Olympus DP71 digital camera and Olympus DP Controller 3.2.1.276 imaging software.

**AV Canal Explant Assay**

The AV canal explant assay was performed to compare endocardial cell migration in E10.5 WT and KLF2-/- hearts. Sterile conditions were maintained while preparing the collagen matrix, dissecting the AV canal and culturing the explants for up to 72 hours.

Collagen matrix was prepared using Type 1 Rat Tail Collagen, High Concentration (BD Biosciences, Bedford, MA; Lot # 21114). Collagen concentration for the given lot was 9.18mg/ml. 500µl aliquots of collagen were prepared and stored at 4°C. This prevents gelling of collagen before use and thus any errors in the collagen concentration. Collagen, sterile 10X PBS, dH₂O and 1N NaOH were placed on ice 15 minutes before the matrix was prepared. In the final solution, the concentration of PBS was adjusted to 1X and that of collagen was adjusted to 1mg/ml using dH₂O. 0.023ml of 1N NaOH was added per milliliter of Collagen. While making the solution, the collagen was added last, the solution was vortexed and immediately poured in the well. Approximately 2ml collagen matrix was poured per well in a 12 well plate. It takes about 15 minutes for the matrix to gel. For every new lot of High Concentration Collagen, it is important to perform the above steps and ensure that gelling occurs at the required concentration before performing the explant assay.

Embryos were dissected from pregnant mice as described above in a laminar flow hood. Individual embryos were placed in sterile 1X PBS in a sterile Petri dish. Using an Olympus SZ2-ILST dissection microscope, the heart was detached from the embryo and carefully placed on
the Petri dish to expose the atria and the ventricle. The tube-like AV canal between the atrium and the ventricle was detached and cut to expose the endocardium. Figure 2-2 puts the whole embryo, embryo heart and the AV canal in perspective. The AV canal was placed on collagen lumen side down, such that the endocardial region comes in contact with the matrix. Embryo tails were numbered corresponding to the explant well numbers and saved for genotyping. The explants were incubated at 37°C in 5% CO₂ for 10 – 12 hours without any growth medium to facilitate attachment to the matrix. After 10 hours, Medium 199 (HyClone Laboratories, Logan, UT) supplemented with 1% FBS, 0.1% ITS (insulin, transferrin, selenium; Gibco, Grand Island, NY) and 1% Penicillin – Streptomycin (Invitrogen) was added to the explant culture and incubated for about 60 more hours. After 72 hours of incubation, the explants were observed and photographed using an Olympus IX70 inverted microscope with Hoffman Modulation Optics. Hoffman Modulation Contrast system detects phase gradients and converts them into variants of light intensity, giving a 3D effect and better contrast of the unstained objects. The cell counts were performed at a single plane of focus, at which the vast majority of the cells could simultaneously be observed. All of the cells migrated out of the explant, at the given plane, were counted and categorized as either activated (round) cells or transformed (star-shaped) cells. The cell counts for WT and KLF2-/- were compared using Student’s t-test.
Figure 2-2: Dissection of E10.5 AV region. The images show whole embryo, whole heart and the AV canal at E10.5 from left to right. The oval shape indicates whole heart in the embryo and the AV canal region in the whole heart. Approximate sizes of the whole embryo and heart are indicated in the parentheses. The 3 images are taken at increased magnification from left to right.
Results

*KLF2-/- embryos in the FVB/N genetic background die by E11.5*

Matings between FVB/N KLF2+/- mice resulted in the expected number of embryos of each genotype at E9.5 and E10.5. However, out of 28 embryos from four KLF2+/- matings, no (zero) KLF2-/- E11.5 embryos were obtained (expected frequency = 7). Chi-square analysis was performed to compare the observed and expected frequencies (Table 2-1), and it was determined that the number of KLF2-/- embryos was significantly less than expected (p = 0.0044). FVB/N KLF2-/- embryos die by E11.5, sooner than KLF2-/-embryos in a mixed genetic background, which die by E14.5 (Huddleson et al., 2005; Kuo, Veselits et al., 1997; Lee et al., 2006). This suggests that modifier alleles in FVB/N affect the KLF2-/- phenotype. It is intriguing that E10.5 animals have expected numbers of KLF2-/- embryos whereas the total number of alive as well as dead embryos observed at E11.5 KLF2-/- was less than expected. The possible explanation is that the mutant embryos get rapidly resorbed overnight and hence KLF2-/- is not detected. The male animals used to obtain E10.5 and E11.5 embryos were not the same. However both males used to obtain E11.5 animals contributed to one partially resorbed KLF2-/- embryo. This indicates that none of the male parents were mis-genotyped, and were actually KLF2+/- . This does not exclude the possibility that one of the female parents was mis-genotyped.
As described previously in Fig. 1-5, the FVB/N KLF2-/- embryos showed an accumulation of cells lining the AV canal region, and these cells formed multiple disorganized layers (n = 4), compared to FVB/N WT. To quantify this observation, cell counts were performed to determine the number of cells lining the AV canal (Fig. 2-3A). There are 2-fold more cells lining the AV canal in KLF2-/- than in WT embryos. The Student’s t-test indicates that this is a significant difference with a p-value of 0.0078. In order to investigate whether the accumulation of cells constricted the AV canal, E9.5 KLF2-/- AV canal volumes were measured (n=4). As expected, KLF2-/- AV canal volumes were approximately 3-fold lower than WT, representing a significant difference with a p-value of 0.0028 (Fig. 2-3B). Thus, FVB/N KLF2-/- hearts have narrower AV canals compared to FVB/N WT hearts. However, overall the heart appears smaller in FVB/N KLF2-/- than WT embryos. Thus heart volume is likely consistent with an overall

<table>
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<th>Stage</th>
<th>Total viable embryos</th>
<th>WT</th>
<th>KLF2+/—</th>
<th>KLF2-/-</th>
<th>Chi-square p-value</th>
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<td>8</td>
<td>6.5</td>
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<tr>
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<td>27</td>
<td>12 (0)</td>
<td>6.75</td>
<td>15 (1)</td>
<td>13.5</td>
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Parentheses indicate number of dead embryos at E11.5. O is observed; E is expected. Asterisk indicates a statistically significant difference between O and E.

E9.5 FVB/N KLF2-/- mice have increased number of cells lining the AV canal

Table 2-1: Number of embryos observed and expected from FVB/N KLF2+/- matings
change in heart morphology. AV morphological defects were not reported in KLF2-/ hearts from mice in a mixed genetic background (Huddleson et al., 2005; Lee et al., 2006).

Figure 2-3: E9.5 FVB/N KLF2-/ hearts show increased number of cells lining the AV canal and consequently a constricted AV canal, compared to WT. A) Bar chart representing the number of cells/mm² lining the AV canal of E9.5 WT and KLF2-/ embryos. The Student's t-test indicates that the number of cells is significantly greater in KLF2-/ than in WT (p = 0.0078). B) Bar chart comparing E9.5 WT and KLF2-/ AV canal volumes. KLF2-/ AV canal shows significant decrease in volume, compared to WT (p = 0.0028). n = 4
E10.5 FVB/N KLF2/- embryos have hypoplastic AV endocardial cushions and other cardiac abnormalities

To examine the KLF2/- AV endocardial phenotype at a later stage of development, light microscopy was performed at E10.5 (n = 3). The cardiac abnormalities in FVB/N KLF2/- heart are more severe at E10.5 than at E9.5. The cells in the AV canal region and endocardial cushions in FVB/N KLF2/- embryos (Fig. 2-4C and 2-4D) are disorganized compared to somite matched WT controls (Fig. 2-4A and 2-4B). In E10.5 FVB/N KLF2/- embryos, the AV cushions are hypocellular (Fig. 2-4D at *) compared to WT, which are highly populated with mesenchymal cells (Fig. 2-4B at *). In FVB/N KLF2/- embryos, the endocardial cells are evidently unable to transform into mesenchymal cells and migrate into the cushions, and therefore endothelial-like squamous cells accumulate lining the AV canal. Moreover, the E10.5 FVB/N KLF2/- heart has only one atrium (Fig. 2-4C), whereas somite-matched WT hearts have a left and right atrium at this time point (Fig. 2-4A), indicating that there is also an atrial septal defect in the septum primum in the mutants. Additionally, in the E10.5 FVB/N KLF2/- heart (Fig. 2-4C), the myocardium is thinner than in the WT heart (Fig. 2-4A), as previously reported (Lee et al., 2006). KLF2 is not expressed in the myocardium, thus its effect on myocardial development must be indirect.

E10.5 endothelial-specific Tie2-cre KLF2/- (Tie2-cre KLF2/-) hearts show hypocellular endocardial cushions, disorganized AV canal regions, and delayed atrial septal formation (Fig. 2-4G and 2-4H), similar to the traditional KLF2 KO. Using this model, the deletion of the KLF2 gene in the heart is quite complete at approximately 84% (unpublished data). Negative control littermates having the floxed KLF2 gene without Tie2-cre are unaffected, as expected and shown in Fig. 2-4E and 2-4F. This suggests that KLF2 has an endothelial cell-autonomous role in the AV cushion region.
The E10.5 FVB/N KLF2-/- heart morphological phenotype is more severe than that previously reported by Lee et al. for mice in a mixed genetic background, which had only myocardial thinning (Lee et al., 2006). To confirm that the KLF2-/- phenotype varies in different genetic backgrounds, matings were carried out to obtain KLF2-/- embryos in a controlled genetic background that is 50% FVB/N and 50% C57BL/6 (mixKLF2-/-). Light microscopy studies on E10.5 mixKLF2-/- embryos indicate that the AV canal and cushion morphology in these embryos (Fig. 2-4K and 2-4L) is comparable to somite- and genetic background-matched WT embryos (Fig. 2-4I and 2-4J). The endocardial cushions in mixKLF2-/- embryos do not have a drastic reduction in mesenchymal cells (Fig. 2-4L). However, mixKLF2-/- embryos (Fig 2-4K) have thinner myocardium than mixWT (Fig. 2-4I), as previously reported (Lee et al., 2006). C57BL/6 KLF2-/- hearts (Fig. 2-4O and 2-4P) have apparently normal AV cushions like mixKLF2-/- and C57BL/6 WT hearts (Fig. 2-4M and 2-4N), and thinner myocardium like the mixKLF2-/- and FVB/N KLF2-/- hearts. C57BL/6 KLF2-/- hearts (Fig. 2-4O) have atrial septal defects similar to those observed in FVB/N KLF2-/- hearts (Fig. 2-4C), but not found in mixKLF2-/- hearts(Fig. 2-4K).

To quantify the mesenchymal cell hypocellularity of the AV endocardial cushions, cell counts were performed for E10.5 WT and KLF2-/- embryos in the FVB/N, C57BL/6 and mixed genetic backgrounds, and for the FVB/N Tie2-cre KLF2-/- embryos (Fig. 2-5). As expected, FVB/N KLF2-/- and Tie2-cre KLF2-/- embryos showed highly significant (p < 0.001) decreases in the number of mesenchymal cells in the endocardial cushion tissue associated with the AV canal, compared to controls. Interestingly, C57BL/6 KLF2-/- embryos also showed a statistically significant (p = 0.03) decrease in the mesenchymal cell count, suggesting that there is some role for KLF2 in EMT regardless of the strain.
Figure 2-4: E10.5 FVB/N but not mix and C57BL/6 KLF2-/- atrioventricular endocardial cushions are hypoplastic and disorganized. (A) and (B) are micrographs of FVB/N WT heart; (C) and (D) are micrographs of FVB/N KLF2-/- heart. The light micrographs, A and C (magnification 100X), show the structure of the E10.5 heart including the atrial (At) and the ventricular chambers (V) and B and D show the endocardial cushion regions that are in the boxes in A and C respectively, magnified at 200X. The red dashed lines indicate the positions of the hypoplastic FVB/N KLF2-/- AV cushions in D, compared to normal AV cushions in H and L. (G) and (H) are micrographs of Tie2-cre KLF2-/-, and (E) and (F) are WT littermate controls without Tie2-cre; all are in the FVB/N background. (I) and (J) are micrographs of mixWT; (K) and (L) are micrographs of mixKLF2-/- hearts. (M) and (N) are micrographs of C57BL/6 WT; (O) and (P) are micrographs of C57BL/6 KLF2-/- hearts. Mes: Mesenchymal cells; Endo: Endothelial cells; Ery: Erythroid cells; Myo: Myocardium. n = 3-5 hearts for histological staining, and n = 3 hearts for mesenchymal cell counts. WT and KO embryos for each background are somite matched. Asterisks* indicate AV endocardial cushion region. Arrowheads
Figure 2-5: E10.5 KLF2-/- AV cushions show a decrease in the number of mesenchymal cells. Bar chart representing the number of mesenchymal cells/mm² in the endocardial cushion tissue associated with the AV canal. Counts were performed of all cells in a single central section from E10.5 WT or KLF2-/- hearts in all three genetic backgrounds and for Tie2-cre KLF2-/- hearts. Student’s t-test indicates that the number of mesenchymal cells is decreased in FVB/N KLF2-/- (p < 0.001), Tie2-cre KLF2-/- (p < 0.001) and C57BL/6 KLF2-/- (p = 0.032), compared to WT. FVB/N and C57BL/6 KLF2-/- AV cushions show significant decrease in the mesenchymal cells, however FVB/N KLF2-/- has a more severe decrease compared to the C57BL/6 KLF2-/- (p = 0.00058).
Cells accumulated in the FVB/N KLF2-/- AV canal have endothelial character

To further define the role of KLF2 in EMT, the cells accumulating in the FVB/N KLF2-/- AV canal were studied to determine the cell type. The cells could potentially be endocardial cells or mesenchymal cells based on their location and developmental age of the embryo. Light and electron microscopy showed squamous morphology of these cells at E9.5, suggesting them to be endocardial cells. Thus immunohistochemical staining was performed using the endothelial specific PECAM (CD31) antibody to verify the cell type. The cells accumulated in the FVB/N KLF2-/- AV canal are CD31 positive, indicating that they have endothelial characteristics (results from two embryos shown in Fig. 2-6C- 2-6F). The WT positive control has an organized layer of CD31 positive endothelial cells lining the AV canal (Fig. 2-6A and 2-6B). A negative control, reacted only with secondary antibody, had no staining. Expression of PECAM1 mRNA was significantly higher in FVB/N KLF2-/- than in FVB/N WT AV canals as shown by quantitative reverse transcriptase-PCR (qRT-PCR, Fig. 5G). This suggests that there is an increased number of cells expressing PECAM1 mRNA, and/or PECAM1 expression per cell is increased in KLF2-/- . However, because an accumulation of endothelial-like squamous cells lining the AV canal is observed in KLF2-/- , there is a high probability that there are a higher number of cells expressing PECAM1 mRNA. These findings support the premise that there is abnormal EMT in the AV cushions of FVB/N KLF2-/- embryos and that the cells abnormally accumulating at the AV canal are endothelial cells that are unable to transform.
Figure 2-6: E10.5 FVB/N KLF2-/- hearts have accumulated endothelial cells lining the AV canal. Immunohistochemistry staining (n = 2) was performed using an endothelial cell specific mouse PECAM (CD31) antibody. A goat anti-mouse secondary antibody conjugated with HRP was used, and reacted with diaminobenzidine (DAB) for detection. Brown coloration of cells indicates a CD31 positive cell type. (A) The cells lining the AV canal in WT are CD31 positive. (B) Higher magnification of WT AV region. (C) and (E) Cells accumulated in the AV canal in two different KLF2-/- hearts are CD31 positive, indicating that they are endothelial cells. (D) and (F) Higher magnifications of KLF2-/- AV regions show accumulation of stained cells. Asterisks* indicate AV endothelial cushion region. A, C and E are 100X magnification; B, D and F are 200X. (G) qRT-PCR shows a 2-fold increase in expression of PECAM mRNA in FVB/N KLF2-/- AV canals compared to FVB/N WT (p = 0.002, n = 7). The amount of PECAM mRNA in WT was designated 100%.
**E10.5 FVB/N KLF2-/- hearts have reduced glycosaminoglycans in the extracellular matrix**

The accumulation of endothelial-like cells in the AV canal and the absence of mesenchymal cells in endocardial cushions in FVB/N KLF2-/- mice suggest that KLF2 regulates EMT during AV cushion formation. Cardiac jelly is a prerequisite for endothelial cell transformation and migration. Glycosaminoglycans form the major component of the cardiac jelly and are essential for endocardial cushion EMT. Therefore, alcian blue staining was performed to stain glycosaminoglycans in WT and KLF2-/- embryos in FVB/N and mixed genetic backgrounds. At E10.5, FVB/N KLF2-/- hearts have a vast reduction of glycosaminoglycans in the cardiac jelly indicated by lack of alcian blue stain (Fig. 2-7B and 2-7E) compared to WT (Fig. 2-7A and 2-7D). This complements the data indicating that KLF2 is required for EMT in the endocardial cushions. Alcian blue staining appears normal in mixKLF2-/- hearts (Fig. 2-7C and 2-7F), indicating that the reduction of GAGs is specific to the FVB/N genetic background. At lower magnification, FVB/N WT (Fig. 2-7A) and Mixed KLF2-/- (Fig. 2-7C) show alcian blue staining in the septum primum between the two future atria. The septum primum is the primordium of the atrial septum. FVB/N KLF2-/- shows absence of septum primum (Fig. 2-7B), as was observed in Fig. 2-4 C. The extracellular matrix in the cranial region and neural tube cross sections seems to be Alcian blue positive in FVB/N KLF2-/- embryos. However it was not quantified to compare with WT embryos. This data suggests that there is a specific effect of KLF2 ablation on the AV canal. This also serves as a positive control for Alcian blue staining in KLF2-/- embryos.
Figure 2-7: E10.5 FVB/N KLF2-/- hearts have abnormal cardiac jelly composition. Alcian blue staining for extracellular matrix and counterstain with nuclear fast red was performed on cross-sections of FVB/N WT and KLF2-/- hearts (n = 3) and Mixed KLF2-/- A, B, C are micrographs at 100X magnification and D,E, F are micrographs at 200X magnification. (A) and (D) WT embryo AV cushions, the nuclei are stained red and the extracellular matrix is stained blue. (B) and (E) FVB/N KLF2-/- AV cushion with decreased blue staining, indicating reduced glycosaminoglycans. (C) and (F) Mix KLF2-/- AV cushions show Alcian blue staining similar to that in WT embryos. At: Atrium; V: Ventricle. Boxes indicate the AV endocardial cushion region.
**A reduced number of mesenchymal cells invade the collagen gel in FVB/N KLF2-/- AV explant assays**

To determine whether the EMT defect is also caused by an abnormality in the endocardial cells in FVB/N KLF2-/- hearts, E10.5 AV explant assays were performed using a collagen gel. FVB/N WT explants underwent EMT and the mesenchymal cells migrated into the collagen matrix during the 72 hour incubation (Fig. 2-8A). Compared to WT explants, KLF2-/- explants showed a significant reduction in the number of mesenchymal cells that migrated into the collagen matrix (Fig. 2-8B and 2-8C). The KLF2-/- explants have at least a 10-fold reduction in the number of transformed cells compared to WT (Fig. 2-8C). In addition, the fraction of migrated cells that are transformed is greater than activated cells in WT explants, but the reverse is true in KLF2-/- . This suggests that a defect in the endocardial cells, as well as in the cardiac jelly composition, is responsible for abnormal EMT in the FVB/N KLF2-/- AV cushions.
Figure 2-8: E10.5 FVB/N KLF2/- hearts show reduction in transformed mesenchymal cells. (A) and (B) AV canal explants were incubated in vitro on a collagen matrix for 72 hours, and the cells migrating into the matrix were observed. (A) FVB/N WT explants show mesenchymal cells migrating into the collagen matrix (n = 5). Arrows indicate mesenchymal cells. Round cells are activated but not transformed. Stellate cells are activated and transformed into mesenchymal cells. (B) FVB/N KLF2/- explants have less mesenchymal cells migrating into the collagen matrix than WT (n = 5), indicating an EMT defect in the FVB/N KLF2/- endocardial cells. (C) The bar chart indicates percentage of transformed cells in FVB/NWT and FVB/NKLF2/- (n = 3). The number of cells in WT is set to 100%. KLF2/- explants have more than 10-fold less mesenchymal cells in the collagen matrix compared to WT (p < 0.001).
Discussion

Cardiovascular development and morphogenesis is a complex process, involving a number of highly conserved transcription factors and signaling pathways (Olson, 2006). KLF2 plays a multi-faceted role in cardiovascular development. It is expressed in the endocardium of the developing heart. The accumulation of endothelial-like cells lining the AV canal, reduced EMT, delayed atrial septal formation, and the absence of normal cardiac jelly composition are novel phenotypes for FVB/N KLF2-/- mice, and therefore may be related to the earlier embryonic death in the FVB/N genetic background.

The importance of genetic background in cardiac development has been demonstrated in a number of studies. Sakata et al. studied Hey2 deficient mice and observed a spectrum of cardiovascular anomalies that varied in the BALB/c and C57BL/6 genetic backgrounds (Sakata et al., 2006). Astrof et al. studied the role of fibronectin in heart development; a null mutation in the gene results in arrested heart development earlier in 129S4 than in C57BL/6 embryos (Astrof et al., 2007). The current study shows that the role of KLF2 in the morphology and function of the developing heart is also genetic background specific. In the FVB/N background, loss of KLF2 results in an EMT defect in the AV cushion region, delayed formation of the atrial septum, myocardial thinning and death by E10.5. In the C57BL/6 background, KLF2-/- shows delayed atrial septation and myocardial thinning. In a mixed background the major defect in KLF2-/- hearts is myocardial thinning (Lee et al., 2006)

KLF2 is only expressed in endothelial cells of the developing endocardial cushion at E9.5 and E10.5 (Lee et al., 2006). This justifies the morphological defects in the AV region and decreased expression of cardiovascular genes observed in the FVB/N Tie2-cre KLF2-/-
similar to complete FVB/N KLF2-/- and suggests cell autonomous role of KLF2 in cardiac development.

It would be interesting to identify putative effectors downstream of KLF2 that may impact each of these processes in the embryonic heart.
CHAPTER 3: CARDIAC FUNCTIONAL DEFECTS IN KLF2/- EMBRYOS

Summary

Defects in cardiac morphogenesis are often associated with abnormal heart function and blood flow pattern and vice versa [Reviewed in (Bartman & Hove, 2005)]. Evaluating mouse heart function parameters could facilitate an understanding of the causes of the heart defect. The study of developing heart physiology is technically challenging because the embryos are difficult to access. Embryos can be exteriorized before ultrasound imaging (Phoon, Aristizabal, & Turnbull, 2000). However cardiac function and physiological parameters are highly compromised in this invasive procedure. High frequency echocardiography with 40MHz mechanical transducer was used to assess the function of developing mouse heart in utero. Echocardiography of E10.5 FVB/N KLF2/- embryos indicates that they have abnormal heart function compared to wild-type. 2D ultrasound in M-mode was used to observe cardiac output and ejection fraction in mouse embryos. These parameters were significantly higher in FVB/N KLF2/- compared to WT embryos. Pulse Wave Doppler was used to study velocity of blood flow at the descending aorta. The velocity was significantly decreased in FVB/N KLF2/- compared to WT embryos. There was no difference in the heart rate between the two genotypes. The functional abnormalities were observed in FVB/N but not in mice of a controlled mixed genetic background. It is difficult to reconcile the specific abnormalities in the heart function parameters with the morphological defects. However this study shows that the ultrasound technique can be successfully used for non-invasive analysis of cardiac function as early as E10.5.
Introduction

The heart is the first organ to begin mechanical function before organogenesis is even complete. In an embryo, final structure and function of many organs is not required for survival, but defects in heart function are often embryonic lethal. Though the pumping action of the heart varies, the mechanical events are similar in a developing heart and an adult heart. Fluid tends to flow from a region of higher pressure to that of the lower pressure. The principle of the heart function is to create pressures that direct blood flow using this rule. The 3 major mechanical events during the cardiac cycle are – mid-to-late diastole, systole and early diastole. The atrium and ventricle are both relaxed in the mid-to-late diastole. The pressure in the atrium is slightly higher than the ventricle in the beginning and goes on increasing thereby inducing flow from atrium to the ventricle. Systole is the phase of ventricular contraction and ejection of blood. The aortic pressure gradually increases in this phase inducing blood flow from the ventricle to the descending aorta. In early-diastole the ventricular muscle relaxes rapidly and the pressure in the ventricle again falls below the atrial pressure and ventricular filling begins again. After the valves are formed, the valves shut as pressure in the ventricle or aorta increases, avoiding regurgitation (Vander, Sherman, & Luciano, 1980). Before valve development, there is minimal amount of regurgitation even in normal embryos. However unidirectional flow of blood in the developing heart is maintained by a different pumping system than adult heart called 'Liebau Pumping System' in which rhythmic compression of the heart wall at asymmetric positions happens to ensure unidirectional flow [Reviewed in (Manner, Wessel, & Yelbuz, 2010)(Manner et al., 2010)].

Studies of physiological parameters of a developing heart are technically challenging because the embryos are difficult to access and are not static. In the past, embryos used to be dissected out and then subjected to ultrasound imaging (Phoon et al., 2000). However
this severely compromises the physiological activity of the heart and would question reliability of the cardiac function data obtained. High frequency Doppler Echocardiography (DE) is now used to analyze adult and embryonic hearts in mammals. The principle of DE is that sound waves are compressed, as the source of sound moves towards an observer, and are therefore higher in frequency than those emitted when the source is travelling away from the observer. The change in frequency of the sound is proportional to the velocity of the target in relation to the source of the sound. DE involves the emission of ultrasound waves of known velocity which are reflected from interfaces and return to the transducer. The equipment detects changes in the frequency of the reflected sound in comparison to the emitted sound. In DE, the most important interfaces which reflect the sound are red blood cells (RBCs). Thus, if the emitted ultrasound is reflected off RBCs moving towards the transducer the reflected sound will be of a higher frequency (and shorter wavelength) than the emitted sound. The change in frequency (frequency shift) is proportional to the velocity at which the cells are moving towards the transducer. A computer inside the Doppler unit calculates the velocity of the moving blood from the Doppler shift equation. The calculated velocity is displayed on a velocity/time graph with blood flow towards the transducer displayed above a baseline and flow away displayed below it. This form of display is known as spectral Doppler.

The advanced version of conventional Doppler system is pulse-wave Doppler (PWD). PWD sends a pulse of known, short duration, and then records returning echoes for a limited period some time later. By limiting the period during which returned echoes are detected, the distance from the transducer to the targets which return the echoes is known. The 'gated' period can be displayed on the screen as a small box known as the sample volume. This corresponds to the area of the heart from which the Doppler signals are received. The time taken for the PWD sound-wave to reach the near end of the sample volume can be termed T1. The time taken for it to reach the far end of the sample volume can be termed T2. The
machine starts to listen to returning echoes after $T_1 \times 2$ and stops after $T_2 \times 2$. The sample volume can be guided into specific areas of interest such as the atrial side of atrioventricular (AV) valves to detect regurgitant blood flow, or the right ventricular side of a ventricular septal defect (VSD) to detect blood flowing through the defect. Thus DE can be used to detect blood flow, to identify the direction of flow, and to calculate the velocity of flow.

2D Doppler in the M-mode of high resolution and single ultrasound beam is used to measure structural parameters like ventricular wall size during systole and diastole. The next section explains how these measurements are used to calculate cardiac output and ejection fraction. DE is used to measure the following cardiac functions:

a) Heart rate – Heart rate is the number of cardiac cycles completed in given time. In mouse, the heart begins to pump at approximately E8.5 and is more regular and powerful by E9.5 (Kaufman MH & Bard JBL, 1999). It is important to measure the heart rate accurately under normal conditions, since other physiologic parameters are influenced by it. The embryonic heart rate is not under neuronal control (Clark & Hu, 1990). A few studies suggest that it is controlled by regional ventricular-vascular coupling or is under genetic control (Keller, 1997).

b) Stroke volume – Stroke Volume is the volume of blood ejected during each ventricular contraction. It is calculated as:

$$SV = EDV - ESV$$

$SV = $ Stroke Volume; $EDV = $ End Diastolic Volume; $ESV = $ End Systolic Volume.

Volumetric measurements of the heart are routinely obtained from the ventricular wall dimensions at systole and diastole. Stroke volume increases during development as
the size of the heart increases. The ventricles never completely empty in embryonic heart and thus there is usually huge variation in the stroke volume in normal embryos as well.

c) Cardiac Output – The volume of blood pumped by the embryonic heart per unit time. Cardiac output is a product of stroke volume and heart rate.

\[ CO = (EDV - ESV) \times HR \]

CO = Cardiac Output; EDV – ESV = Stroke volume; HR = Heart Rate

In developed heart with 4 chambers, it is the volume of blood pumped by each ventricle and not the total of the two ventricles. Since stroke volume increases with embryonic growth so does cardiac output.

d) Ejection fraction – The fraction of blood pumped out of the heart with each heart beat or cardiac cycle. Ejection fraction is the ratio of stroke volume to end diastolic volume.

\[ EF = \frac{(EDV - ESV)}{EDV} \]

EF = Ejection Fraction; EDV – ESV = Stroke Volume; EDV = End Diastolic Volume

e) Aortic velocity – The peak velocity of blood during ejection at the aorta. Pulsed- wave Doppler is used. The Doppler frequency is determined using a normal control heart and velocity is calculated from it using the following Doppler equation:

\[ \Delta f = 2f_o(Vlc)\cos\theta \]

\( \Delta f = \text{Doppler Frequency}; \ Vl = \text{Velocity of each red cell}; \ f_o = \text{Ultrasonic frequency, 40MHz of the mechanical transducer}; \ c = \text{Speed of sound in blood (1540 M/s)}; \ \theta = \text{Angle between the sound beam and the direction of flow} \)
**Methods**

*Echocardiography preparation*

KLF2+/- adult mice were mated to obtain E10.5 embryos. Non-invasive *in utero* fetal ultrasound using a VisualSonics Vevo 770 System and 40MHz mechanical transducer (VisualSonics) was performed on 33 E10.5 embryos. For each embryo, blood flow parameters, including heart rate, blood flow velocities and volumes were measured. Physiological parameters such as heart rate and stroke volume show diurnal variations, thus the animals were imaged between 12 and 4pm and were all housed in the same conditions prior to imaging. Three normal pregnancies per genetic background were examined. The embryos were numbered for genotyping based on their position *in utero*, as described previously (Lee et al., 2006). The uterine midline was used as the starting point and the embryos in the left and right horns were named L and R respectively and numbered from bottom up. Thus the embryo closest to the uterine midline on left side was L1, the one above it was L2, etc. At E10.5, the embryos are not static, thus not all the embryos were lying superiorly during the ultrasound. Such embryos required some waiting time of up to a few minutes, which was given, so that all embryos could be assessed. The 40MHz RMV transducer gives an axial resolution of 30μm. The pregnant female mice were anesthetized using pentobarbital (30mg/ kg; ip). The mouse was placed in supine position, the abdomen was shaved using chemical hair remover (Veet) and ultrasound gel was used in the abdomen region to optimize visibility during imaging. Maternal temperature was maintained using heating lamp, if required. Previously described embryo echocardiography protocol was used to generate the protocol used (Corrigan, Brazil, & Auliffe, 2010). Cardiac output was calculated as stroke volume multiplied by heart rate and expressed as beats per minute. 2D imaging was used to view the 3 or 4 chamber heart and measure left ventricle ejection fraction. To statistically compare the values in WT and KLF2-/-, the Student’s t-test was used.
Measurement of Cardiac Function

The scanning modalities used were M-mode, B-mode and Pulse-Wave Doppler (PW Doppler) Mode. M-mode imaging operates at high temporal resolution of 1000 frames per second along a single ultrasound beam. The resolution and the number of frames captured, thus allow observing the ventricular wall size and dimension during systole and diastole. Software analysis tool associated with Vevo – 770 M-mode quantifies the key function parameters like cardiac output and ejection fraction, based on wall dimensions. B-mode is a 2 dimensional ultrasound image display composed of ultrasound echoes represented by bright dots. The brighter the dot, the higher the amplitude of the returned echo signal. This mode is used to physically locate the heart and calculate anatomical parameters like heart rate. PW Doppler mode is used to assess blood flow velocity and direction. The different modes were selected on the keyboard connected to the Vevo 770 machine. A description of cardiac function parameters studied are summarized in Table 1.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Definition</th>
<th>Method of Calculation</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart Rate</td>
<td>Number of heart beats per unit of time</td>
<td>B-mode</td>
<td>bpm</td>
</tr>
<tr>
<td>Cardiac Output</td>
<td>Volume of blood being pumped by the heart at the end of each cycle</td>
<td>M-mode through ventricle; end systolic and diastolic volume</td>
<td>µl/ min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CO = (End diastolic volume – End systolic volume) X heart rate</td>
<td></td>
</tr>
<tr>
<td>Ejection Fraction</td>
<td>Fraction of blood ejected by the ventricle relative to its end diastolic volume</td>
<td>M-mode through ventricle; (End diastolic volume – End systolic volume)/ End diastolic volume X 100%</td>
<td>%</td>
</tr>
<tr>
<td>Aortic flow</td>
<td>Peak velocity blood flow after aortic arch</td>
<td>PW Doppler ; descending aorta</td>
<td>mm/ s</td>
</tr>
</tbody>
</table>
Results

Heart rate is unchanged in the absence of KLF2

To evaluate the function of beating heart, heart rate is the key parameter. It is an indicator of all of the mechanical events as well as the pumping action of the heart. Echocardiography in B-mode was performed on E10.5 KLF2-/- and WT embryos in FVB/N background to measure heart rate. Heart rate was measured as the number of beats per minute. The time difference between consecutive cardiac cycles was measured to determine beats per minute. The embryo is traced during echocardiography. The beating heart can be clearly detected if the embryo lies superiorly (Fig. 3-1A). This can require a few minutes of observation, because E10.5 embryos are not static in the amniotic fluid. No significant difference in heart rate was observed in FVB/N or Mix KLF2-/- compared to WT embryo (Fig. 3-1B). The average heart rates in FVB/N and Mix WT embryos were 420 ± 3.82 bpm and 408 ± 7.58 bpm respectively. The average heart rates in KLF2-/- in FVB/N and Mix background were not very different than WT: 418 ± 4.65 bpm and 415 ± 5.85 bpm respectively (Table 3-2).

Heart rate is a sensitive and important physiological parameter since it influences measurement of most of the parameters determining cardiac function like stroke volume, cardiac output and ejection fraction. The low standard deviation for each group indicates statistical reliability of the data. Student’s t–test was performed to compare average heart rates among KLF2-/- and WT embryos in both the genetic backgrounds (n = 5; p > 0.01).
Figure 3-1: Heart Rate in E10.5 mouse embryos. A) indicates B-mode echocardiography image of an E10.5 WT embryo. The embryo is positioned rightly, with the heart (red box) lying superior to the body and thus easily accessible. Red arrow indicates head of the embryo. The red circle at the bottom indicates the heart rate read out, which is 319 bpm in this case. B) is a bar graph representing heart rate in FVB/N WT, FVB/N KLF2/-/-, Mix WT and Mix KLF2/-/- embryos at E10.5. The heart rate is not significantly different in KLF2/-/- compared to WT in FVB/N and Mix genetic background, p > 0.01. Error bars indicate standard deviation. n = 5.
Effect of KLF2 KO on Cardiac Output and Ejection Fraction

To assess systolic and diastolic function of the embryonic heart physiological parameters like cardiac function and ejection fraction were assessed. Echocardiography in M-mode was performed on E10.5 KLF2-/− and WT embryos in FVB/N and Mix genetic background.

Cardiac output is the volume of blood pumped out of the heart in a given time. It is a product of stroke volume and heart rate. Analysis software for Vevo 770 was used to measure cardiac output based on changing dimensions of the ventricle wall during systole and diastole (Fig. 3-2A). The average cardiac output in E10.5 FVB/N WT was 207 ± 18.62 µl/min. It was significantly increased to 280 ± 8.16 µl/min in FVB/N KLF2-/−. Student’s t-test was performed to show that the increase in cardiac output was statistically significant with p = 0.005 (n = 5) (Fig. 3-2B). As shown in the previous chapter, KLF2-/− embryos in mixed genetic background do not show AV endocardial cushion and septal defects. Thus it was necessary to study cardiac function in these embryos to see if the functional defect is associated with morphogenetic defect. The average cardiac output in E10.5 Mix KLF2-/− was 215 ± 5.77 µl/min and was comparable to 202 ± 21.68 µl/min in Mix WT. Thus KLF2-/− embryos in Mix genetic background did not show high output compared to Mix WT.

Ejection fraction (EF) is another measure of systolic and diastolic function. It is represented as the percentage of difference between end diastolic volume (EDV) and end systolic volume (ESV) end diastolic volume. Thus EF = EDV – ESV / EDV *100. Ejection fraction is measured similar to cardiac output (Fig. 3-2A). As expected, after looking at the cardiac output data, average EF in E10.5 FVB/N KLF2-/− embryos (94 ± 1.79 %) was significantly higher than FVB/N WT embryos (81 ± 3.2 %). Student’s t-test showed that the
averages were statistically different with $p = 0.037$ ($n = 5$). Like cardiac output, average EF was not statistically different in Mix KLF2-/- (83 ± 2.85 %) compared to Mix WT (80 ± 3.02 %) at E10.5 ($n = 5$).

Figure 3-2: Cardiac output and Ejection Fraction in E10.5 embryos. A) indicates M-mode echocardiography image of E10.5 embryo heart – cross section of the ventricle wall taken using Vevo 770. The red arrow in the top image points out at the left ventricle where the measurement is taken at. The waves in the bottom indicate inner and outer ventricular wall at systole and diastole. At systole the ventricle contracts, decreasing the distance between the two layers and at diastole it relaxes increasing the distance. Depth analysis is performed using M-mode to measure the change in ventricular wall dimensions. The blue writing is a read out of stroke volume, cardiac output, ejection fraction, etc. generated using software analysis tool from the ventricular wall dimensions. Bar graphs represent B) Cardiac Output and C) Ejection fraction in FVB/N WT, FVB/N KLF2-/-, Mix WT and Mix KLF2-/- at E10.5. FVB/N KLF2-/- embryo shows significant increase in cardiac output and ejection fraction compared to FVB/N WT ($p = 0.005$ and $p = 0.0037$, respectively). $n = 5$. Asterisk indicates statistical significance.


*Descending aorta velocity changes in absence of KLF2-/-*

In the previous chapter, it was shown that in absence of KLF2-/- in FVB/N genetic background at E9.5 and E10.5 there are cells accumulating in the AV canal. This reduces the AV canal volume as observed in Fig. 2-XY. To study the effect of AV constriction on the volume and velocity of blood flowing out of the heart, descending aorta velocity was observed for E10.5 KLF2-/- and WT embryos. Doppler measures blood velocity by detecting the difference in frequency between an emitted burst of ultrasound (40MHz) and the returning echoes from the moving blood. The descending aorta velocity is the peak velocity at the aortic ejection.

FVB/N KLF2-/- shows smaller peak of blood flow at the end of every cardiac cycle in the ultrasound micrograph compared to FVB/N WT (Fig. 3-3 A and B). WT embryo (Fig. 3-3 A) shows some amount of regurgitation which is occasionally observed in embryos at this embryonic age due to absence of valves. Measurement of Descending Aorta velocity showed that FVB/N KLF2-/- embryo hearts had an average velocity of 59 ± 4.14 mm/s lesser than FVB/N WT average of 98 ± 7.3 mm/s. The reduction in dorsal aorta velocity was statistically significant as tested by Student’s t–test (p = 0.01, n = 5.). There was no significant difference in the velocity of blood in the mix WT and mix KLF2-/- embryos. Thus this cardiac functional parameter is also dependent on the genetic background.
Figure 3-3: Descending Aorta Velocity in E10.5 embryos. A) and B) show a Pulse Wave Doppler Echocardiography image of descending aorta blood flow in FVB/N WT and KLF2-/- heart respectively. The position of the embryo heart varies, since it is not static. Thus the direction of flow in the above images is opposite to that in the adult Doppler. The blue line indicates the peak flow at every cycle. The Y-axis on the left is the Doppler Frequency whereas on the right is velocity of blood. The Doppler frequency is manually determined using normal control. Velocity is given as a read out using software analysis tool. Smaller peak is seen in KLF2-/- (B) compared to WT (A). C) Bar graph represents Descending Aorta velocity in FVB/N WT and KLF2-/- and Mix WT and KLF2-/- FVB/N KLF2-/- shows significant decrease the DA velocity (p = 0.01). n = 5. Asterisk indicates statistical significance.
Table 3-2: Cardiac Function Analysis of E10.5 embryos

<table>
<thead>
<tr>
<th>Parameter</th>
<th>FVB/N WT</th>
<th>FVB/N KLF2/-</th>
<th>Mix WT</th>
<th>Mix KLF2/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart Rate (bpm)</td>
<td>420 ± 3.82</td>
<td>418 ± 4.65</td>
<td>408 ± 7.58</td>
<td>415 ± 5.85</td>
</tr>
<tr>
<td>Cardiac Output (µl/s)</td>
<td>207 ± 18.62</td>
<td>280 ± 8.16*</td>
<td>202 ± 21.68</td>
<td>215 ± 5.77</td>
</tr>
<tr>
<td>Ejection Fraction (%)</td>
<td>81 ± 3.2</td>
<td>94 ± 1.79*</td>
<td>83 ± 2.85</td>
<td>80 ± 3.02</td>
</tr>
<tr>
<td>Descending aorta velocity (mm/s)</td>
<td>98 ± 7.3</td>
<td>59 ± 4.14*</td>
<td>93 ± 1.51</td>
<td>97 ± 2.61</td>
</tr>
</tbody>
</table>

*Asterisk indicates statistical significance.
Discussion

The functional defects in FVB/N KLF2-/- and their absence in Mix KLF2-/- correlate with the morphological AV endocardial cushion region and septal defects described in the previous chapter. However there are conflicting cardiac function observations in FVB/N KLF2-/- embryos. Firstly, the cardiac output in these embryos is high; however the heart rate is normal. Cardiac output is a product of heart rate and stroke volume. Thus our observations, in mutants, suggest that the stroke volume is high in the embryos which would be an outcome of higher ventricular contraction. An increase in the sympathetic activity of the myocardial fibers releases norepinephrine that causes increase in the strength of myocardial contraction (Vander, Sherman, & Luciano, 1980). Another conflicting observation is that mutant embryos show an increase in cardiac output but a decrease in aortic velocity. This could be caused by AV regurgitation. It was not possible to accurately measure regurgitation in the E10.5 AV canal because of the continuous movement of the embryo. An increase in the cardiac output is usually an indicator of arterial stiffness (Mackenzie et al., 2002). The lower aortic velocity indicates that the heart might actually be working to avoid arterial stiffness in the KLF2 mutants. It is thus difficult to reconcile the specific abnormalities in the heart parameters with the morphological defects. The defects in heart function in E10.5 FVB/N KLF2-/- mice are likely not related to myocardial thinning, which occurs in both FVB/N KLF2-/- and mixKLF2-/- embryos.

A previous study on the role of KLF2 in heart development showed that KLF2-/- embryos in undefined mixed genetic background had high cardiac output and ejection fraction from E11.5 onwards. E10.5 KLF2-/- embryos were tested in this study (Lee et al., 2006). Our work shows that FVB/N KLF2-/- has functional defects at earlier stage of development in not only cardiac output and ejection fraction but also in aortic velocity. This
suggests that absence of KLF2 in FVB/N genetic background has more severe effects on cardiac function than in just cardiac morphogenesis.

Murine embryonic echocardiography at the stage as early as E10.5 was rarely attempted in the past. The main reasons being that: 1) the embryos are difficult to access; 2) they are not static and keep floating in the amniotic fluid; 3) there is a risk of getting a false reading from mother’s vasculature around the abdomen area. The techniques used in the past to study cardiac functions were invasive sensors inserted in the heart by surgical procedure (Hartley, Hanley, Lewis, & Cole, 1978). This disturbed the normal physiology of the animal. The other concerns were size of the sensor, accuracy, fidelity and calibration [Reviewed in (Kass, Hare, & Georgakopoulos, 1998)]. These techniques were almost impossible to use in embryos. The next generation techniques were also invasive but facilitated the study of embryonic hearts. The embryos were dissected out and exposed to sensors or ultrasound bioimaging (Phoon et al., 2000). Exteriorizing the embryo is harmful and definitely affects cardiac functions like heart rate and stroke volume. Our study shows that noninvasive high frequency ultrasound echocardiography can be used to accurately measure cardiac function and assess cardiac structure of the developing mouse heart, as early as E10.5. A study was previously performed to observe cardiac function parameters in normal mouse embryos from E10.5 until the juvenile phase (Corrigan et al., 2010). However, our study of KLF2-/- embryos is the first to use the technique to study the etiology of a cardiac defect in E10.5 mutant embryos.
CHAPTER 4: INVESTIGATING THE MOLECULAR MECHANISMS FOR CARDIOVASCULAR DEFECTS IN KLF2-/- MOUSE EMBRYOS

Summary

KLF2 is a transcription factor expressed in endothelial cells of the developing heart (Lee et al., 2006). Transcription factors regulate expression of a variety of genes and have the capacity to activate or inactivate cellular signaling pathways. Hence there is a high probability that KLF2 is responsible for cardiac development by regulating one or more downstream target genes. Identifying the critical targets is thus instrumental in obtaining a complete understanding of how the KLF2 mutation results in the cardiac abnormality. The genes that play important roles in AV cushion EMT, cardiac jelly synthesis and septal formation were shortlisted based on the literature. The expression of these genes was studied in WT and KLF2-/- whole embryonic hearts and/or specifically in AV regions. KLF2 ablation results in reduced Sox9, UDP-glucose dehydrogenase (UGDH), Gata4 and Tbx5 mRNA in traditional KLF2-/- as well as endothelial specific KLF2-/- AV regions in FVB/N background. Gata4, Tbx5 and Sox9 are cardiovascular transcription factors that play an important role during development. UGDH encodes an enzyme required for synthesis of cardiac jelly. KLF2 binds to the Gata4, Tbx5 and UGDH promoters in chromatin immunoprecipitation assays, indicating that KLF2 could directly regulate these genes. These data partially explain the molecular mechanism by which KLF2 ablation causes the cardiac defects. Mix KLF2-/- AV regions did not show any change in expression of Gata4, Tbx5, Sox9 and Ugdh mRNA, correlating with the absence of EMT and septation abnormality in these mice.
Introduction

During mouse heart development, localized swellings of the endocardial layer arise at approximately embryonic day 9.5 (E9.5), and form the endocardial cushions of the atrioventricular (AV) canal and the outflow tract. The endocardial cushions are formed by endothelial to mesenchymal transformation (EMT). During EMT, AV endocardial cushion cells undergo hypertrophy, loss of cell-cell contacts, lateral mobility, formation of mesenchymal-like cell processes (filopodia), and migration into the cardiac jelly (Reviewed in (Lim & Thiery, 2012)). Normal cardiac jelly synthesis is required for endothelial cells to transform and to migrate, absence of which results in hypoplastic endocardial cushions (Krug, Runyan, & Markwald, 1985a). Extensive remodeling and proliferation of the endocardial cushions occurs to form the adult heart valves and atrial and ventricular septa. By E10.5, the right and left atria have divided (Nakajima, Yamagishi, Hokari, & Nakamura, 2000b); the AV endocardial cushion region also plays an important role in septation of the heart (Reviewed in (Person et al., 2005)). Through studies with mouse genetic models, a number of molecules and signaling pathways have been identified to play important roles in cardiac jelly synthesis, EMT and septation during endocardial cushion development (Fig. 4-1).

Notch1 is expressed in endocardial cells from E8.0 and is known to play a pivotal role in cardiac cushion EMT (Grego-Bessa et al., 2007; High & Epstein, 2008). Notch1-/- embryos die by E10.5 due to a number of cardiac and vascular abnormalities (Timmerman et al., 2004). Notch1-/- embryos have hypocellular cardiac cushions, with respect to mesenchymal cells, and reduced trabeculation of the ventricular wall (Swiatek, Lindsell, del Amo, Weinmaster, & Gridley, 1994). Hesr1 and Hesr2 are targets of the Notch1 signaling pathway. Double knock-out (KO) embryos for Hesr1 and Hesr2 show a cardiac phenotype similar to Notch1-/- (Kokubo, Tomita-Miyagawa, Hamada, & Saga, 2007b). Bone
morphogenetic protein 2 (BMP2) is expressed in myocardium from E8.0 through the time that endocardial cushions begin to form at E10.5, and then it is expressed in cushion mesenchymal cells (Kokubo, Tomita-Miyagawa, Hamada, & Saga, 2007b). BMP2/- mice die by E8.5; however an AV myocardium conditional KO of BMP2 showed that BMP2/- cardiac cells are incapable of EMT induction and cardiac jelly accumulation (Sugi, Yamamura, Okagawa, & Markwald, 2004a). Thus BMP2 signaling plays an important role in cardiac EMT via myocardial signaling, even though BMP2 is not expressed in endocardial cells. Msx1 and Msx2 are closely related downstream effectors of the BMP signaling pathway, expressed in heart endocardium and myocardium from E9.5. Deficiency of Msx1 and Msx2 in mouse embryos results in hypoplastic AV cushions, with respect to mesenchymal cells, by E10.5 (Ma, Lu, Schwartz, & Martin, 2005b). Thus Msx1/2 form a link between myocardial and endocardial signaling during AV cushion development. Double KO Msx1 and Msx2 embryos show reduced expression of mesenchymal hyaluronan synthase 2 (Has2) (Chen, Ishii, Sucov, & Maxson, 2008b), an enzyme that produces hyaluronan. Hyaluronan is a major glycosaminoglycan (GAG) component of the cardiac jelly. Has2 is expressed in endocardium, myocardium and cardiac mesenchyme from E9.5. Has2/- embryos have an absence of EMT and lack of endocardial cushions (Camenisch et al., 2000). The above molecules are involved in one or more of the signaling pathways during cardiac development.

A few other molecules are cardiac transcription factors that play an important individual role in cardiac development. Sox9 is one such transcription factor expressed in WT embryonic endocardial cells from E9.5 onwards. Sox9 -/- embryos die by E12.5, and have aberrant migration of endocardial cells, which causes hypoplastic cushions (Akiyama et al., 2004). Other functions of Sox9 in cardiac development are precursor cell proliferation and extracellular matrix organization (Lincoln et al., 2007). T-box 5 protein (Tbx5) is a transcription factor required for cardiac cushion and septum formation. Tbx-5 mutations
cause Holt-Oram syndrome, an autosomal dominant disorder characterized by skeletal and cardiac defects. Tbx5 is expressed in endocardial cells from E9.0 onwards (Basson et al., 1997; Basson et al., 1999; Bruneau et al., 2001). Gata4 is another transcription factor. Gata4-/- results in AV endocardial cushions hypocellular to mesenchymal cells similar to KLF2-/- from E9.5 onwards (Rivera-Feliciano et al., 2006). Interestingly, the Tbx5 and Gata4 proteins physically interact during cardiac development. In mice with this Gata4 mutation and a null allele for Tbx5, Gata4+/Tbx5+/-, there is normal EMT but defective remodeling, resulting in septal defects (Garg et al., 2003; Maitra et al., 2009). UDP-Glucose Dehydrogenase (UGDH) is an enzyme required for the conversion of UDP-Glucose to UDP-Glucuronic acid, which is further used in the biosynthesis of HA, GAGs, heparin sulphate and chondroitin. Thus it plays an important and an early role in cardiac jelly synthesis (Clarkin et al., 2011).

Endothelial nitric oxide synthase (eNOS/ NOS3) is an enzyme that generates nitric oxide a vasoprotective molecule in adult endothelial cells. eNOS is also expressed in the developing heart in mice from E9.0 (Bloch et al., 1999). NOS3-/- animals die post-natally due to pulmonary congestion and alveolar edema. These embryos also have congenital atrial and ventricular septal defects. NOS3-/- embryos show increased in cardiomyocyte apoptosis by E12.5 (Feng et al., 2002). NOS3 is a KLF2 target gene, known to be positively regulated as a response to fluid shear forces. Studies in HUVEC cell line, using luciferase assays, have shown that KLF2 directly regulates eNOS expression. Promoter deletion and mutational analysis identified a single KLF2 binding site required for KLF2 to activate the eNOS promoter (SenBanerjee et al., 2004). Both the genes together play an anti-inflammatory, atheroprotective role in adult endothelial cells.

KLF2 is a transcription factor expressed in the endocardial cells during development. The roles of KLF2 in AV cushion EMT, cardiac jelly synthesis and atrial septation reported
here were not previously known. Thus KLF2 is a novel player in cardiac development. In the previous chapters we described the abnormalities during cardiac development in absence of KLF2. KLF2 might regulate any of the cardiac genes described above. Thus to understand the biology of and thereby treat congenital heart and flow defects it is important to know the downstream targets of KLF2 in the developing heart. Gata4 is a particularly interesting putative target, because Gata4 knockout embryos have an AV endocardial phenotype similar to KLF2−/−. Bmp2 signaling pathway is also interesting because the absence of Bmp2 or its downstream molecules results in a lack of cardiac jelly synthesis and an EMT defect similar to KLF2−/−. These and other hypothetical downstream targets were investigated in the following work.
Figure 4-1 Signaling pathways in cardiac development. Blue represents transforming growth factor β (TGFβ) and Bone Morphogenetic Protein (BMP) signaling molecules, through Smad proteins. Pink represents ErbB and Ras Signaling. Yellow represents vascular endothelial growth factor (VEGF)—nuclear factor of activated T-cells pathway (NFAT). Green represents shared (common) genetic signaling proteins/Transcription factors. Molecules in rectangles are ligands; circles are receptors; parallelograms are transcription factors; triangles are carrier protein; diamonds miscellaneous; octagons are component of other protein. Dashed arrows indicate indirect effect. Minus sign indicates inhibitory effect. HB-EGF heparin binding Endothelial Growth Factor, EGFR Endothelial Growth Factor Receptor, BMPR Bone Morphogenetic Protein Receptor, TGFbR Transforming Growth Factor β Receptor, UDPG UDPGlucose, UDPGA UDP-Glucuronic Acid, UDGH UDP-Glucose dehydrogenase, NFM Neurofibromin, CLN calcineurin. [25]
Methods

**Shortlisting candidate KLF2 target genes**

Based on the known roles of KLF2 as a transcription factor, cardiovascular development genes were selected as candidates that may be regulated by KLF2. These potential downstream regulatory elements were examined and ranked based on the following criteria: (1) literature to prove established role in AV cushion EMT, cardiac jelly synthesis and/or atrial septation (2) similar to KLF2-/- phenotype from E9.5 when ablated, (3) presence of a number of potential KLF2 binding sites, CCRCCC, conserved between mouse and humans, in the proximal promoter within 500bp upstream of the transcription start site, (4) higher expression in endocardial cells than to other cell types in E9.5 and E10.5 hearts, (5) published evidence of regulation of the gene by KLF2 in another system.

**RNA extraction and quality assessment**

RNA was isolated from E10.5 whole hearts and AV regions that were dissected as described in Chapter 1. RNA extraction was performed using a ToTally™ RNA kit. Two hundred microliters of Denaturating Solution (ToTally™ RNA isolation kit, Ambion Inc., Austin, TX, USA) was added immediately to the cryotubes containing frozen AV canal tissue upon removal from a -80 °C freezer. This was especially important, because as the tissue thaws, ice crystals will tear cells, releasing RNase into the solution, so denaturant should be present prior to melting so as to inactivate RNase before significant RNA degradation occurs. The tissue was promptly homogenized with a Fisher Scientific Genie2™ Vortex. Once the tissue was completely broken up, 20µl (1/10th volume) of 3M Sodium acetate (ToTally™ RNA kit) was added, and 220µl of Phenol: Chloroform: IAA (ToTally™ RNA kit) was added to the lysate, followed by vortexing. The extract was left on ice for 5 minutes, and then centrifuged in a 1.7 mL centrifuge tube at 11,000 rpm in a Tomy MX-160 High Speed
Refrigerated Microcentrifuge at 4°C for 5 min. The upper aqueous phase was transferred to a new 1.7 mL tube, being careful not to come in contact with the interface. After mixing, starting volume of Acid-Phenol:Chloroform (ToTally™ RNA kit) was added, the tube was vortexed, and was again centrifuged at a speed of 11,000 rpm at 4°C for 5 min. The upper phase was extracted and moved to a new 1.7 mL tube. An equal volume of isopropanol was added and mixed. The solution was stored overnight at -20°C and centrifuged at 11,000 rpm at 4°C for 15 min. A white pellet obtained was saved, the supernatant was discarded, and the pellet washed with 300 μL of 70% ethanol to remove residual salts. The resuspended pellet was centrifuged at 7,500 rpm at 4°C for 10 min, and the supernatant was discarded. The tube was re-spun briefly, and the pellet was air dried before resuspension in 20 μL DNAse – RNase free (Invitrogen, Carlsbad, CA, USA) containing 1:20 dilution of SuperasIn™. One microliter of resuspended RNA was tested for quality and concentration by capillary electrophoresis using Agilent 2100 BioAnalyzer and an Agilent RNA 6000 Pico LabChip (Agilent Technologies, Palo Alto, CA, USA).

**cDNA synthesis**

1 μg of RNA was combined with 1 μL Invitrogen DNase I, 1 μL 10X Reaction Buffer, brought to 10 μL in a microcentrifuge tube with DEPC-treated ultrapure water. This solution was incubated at room temperature for 15 min. at which time 1 μL 25 mM EDTA was added and the solution was heated for 10 min. at 65°C. To the tubes, 4 μL 5X iScript Reaction Mix, 1 μL iScript Reverse Transcriptase, and brought to 20 μL with Nuclease-free water. This mixture was incubated in the following thermocycling program: 5 min. at 25°C, 30 min. at 42°C, 5 min. at 85°C, and held at 4°C until use.

**Quantitative Reverse Transcriptase PCR (qRT-PCR)**
Primers, to amplify the shortlisted genes, were designed using PrimerExpress software (Applied Biosystems) and the sequences of these oligonucleotides are mentioned in Table 4-1. Basic Local Alignment Search Tool (BLAST) was used to confirm the specificity of the primers. qRT-PCR experiments were performed using ABI Prism – 7300 system (Applied Biosystems, Foster City, CA, USA). A SYBR Green absolute program used had following cycles parameters: 1 cycle of 2 min. at 50 °C, 1 cycle 10 min. at 95 °C, 40 cycles of 15 sec. at 95 °C and 1 min. at 60 °C. A dissociation curve was run at the end of the program which consisted of the following: 15 sec. at 95 °C, 30 sec. at 60 °C, and 15 sec. at 95 °C. A standard curve was run using five-fold dilutions of total cDNA (125ng, 25ng, 5ng, 1ng and 0.2ng per well) with each program. All candidate genes were run using SYBR Green reagent. Grand mix was prepared with 12.5 μL Applied Biosystems SYBR Green, 1 μL 10mM candidate gene forward primer, 1 μL candidate gene reverse primer, brought to 20 μL total with HyClone Molecular Grade H₂O per well. This reaction mix was pipetted into a 96-well optical plate and 5μl standard and unknown cDNA samples were added to the respective wells in triplicates. Two dilutions of unknown were used – 25ng and 5ng per well.

The plate was spun down on a Beckman J2-HC centrifuge. A dissociation curve was used for all the qRT-PCR experiments with SYBR Green chemistry. Presence of only one curve confirmed that single product was amplified. CyclophilinA was used as an internal standard to normalize each gene expression. A pre-designed Taqman probe and primer set (Applied Biosystems) was used to measure CyclophilinA mRNA for all the standard and unknown samples. After all of the candidate genes expression was normalized with CyclophilinA expression for all the samples, the relative expression for WT was adjusted to 100 and the expression of KLF2-/- was adjusted accordingly. Standard deviation was used as measure of variance and Student’s t-test was performed compare relative gene expression in WT and KLF2-/-.
Chromatin Immunoprecipitation (ChIP)

ChIP assays were performed using AV regions dissected from E10.5 embryos hearts as described previously in Chapter 2. Briefly, for each biological replicate, approximately 3 X 10^6 cells were pooled from ~8 E10.5 WT AV regions. The dissected AV canals were pooled and washed with PBS followed by trypsinization (0.25% trypsin) at 37°C till tissue is dissociated (~ 3 – 5 minutes; not more than 10 minutes). The cells were then washed with

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tbody>
<tr>
<td>Gata4</td>
<td>CGAGGGTGAGCCTGTATGTA</td>
<td>GCTAGTGCCATTGCTGGAGT</td>
</tr>
<tr>
<td>Tbx5</td>
<td>CAAACTCACCAACAACCACC</td>
<td>GCCAGAGACACCATCTCAC</td>
</tr>
<tr>
<td>Sox9</td>
<td>CGGCTCCAGCAAGAAACAGAG</td>
<td>TGCGCCCACACCATGA</td>
</tr>
<tr>
<td>Bmp2</td>
<td>GTTCGGGCTGAAACAGAGAC</td>
<td>GAATCTCCGGGGTTTTC</td>
</tr>
<tr>
<td>Msx1</td>
<td>CTCCTCAAGCTGCCAGAGAT</td>
<td>GCTTACGGTTCGCTTTGTT</td>
</tr>
<tr>
<td>Msx2</td>
<td>TCGGAAAATTCAGAAGATGGA</td>
<td>GAGGAGCTGGGATGTGGTA</td>
</tr>
<tr>
<td>Has2</td>
<td>AGTCACTATACACAGCCTCAG</td>
<td>CTCCAAACACCTCCAACCATG</td>
</tr>
<tr>
<td>UGDH</td>
<td>CTGCCGGAAGTAGCTCAGTGA</td>
<td>CCTCTTCCTCTGGTAGTTC</td>
</tr>
<tr>
<td>Notch1</td>
<td>TGCCACTATGGTCCTGTAA</td>
<td>GGTTACTGTGCACTCGTT</td>
</tr>
<tr>
<td>Tgfβ2</td>
<td>ATGCCGACTATCTTGCC</td>
<td>CGGTGAACCTCGAACC</td>
</tr>
<tr>
<td>PECAM1</td>
<td>AAGTTTACAAAGAAAAGGAGGAC</td>
<td>ATCCAGGAATCGGGCTTCC</td>
</tr>
</tbody>
</table>

Table 4-1: Primer sequences for candidate KLF2 target gene
equal volume of PBS spun at the speed of 1000rpm at 4°C for 5 minutes and the supernatant is discarded. The cells were cross-linked with 1% formaldehyde in PBS at room temperature for 10 minutes at the end of which 0.125M glycine was added to quench the reaction. The solution was spun at 1000rpm for 8 minutes, supernatant discarded and pellet quick frozen in liquid nitrogen and stored in -80°C till ready to use for the ChiP assay. The cells were then resuspended in 0.8ml Cell Lysis Buffer (10mM Tris, 10mM NaCl, 0.2% NP-40 [pH 8.0] and 3 protease inhibitors) per tube and incubated on ice for 10 minutes and centrifuged for 5 mins. at RT to take supernatant out. The lysed cells were then resuspended in Nuclei Lysis Buffer (50mM Tris, 10mM EDTA, 1% SDS and 3 protease inhibitors) and incubated in it for 10 minutes. ~50µl of the chromatin was saved as unsonicated control and the remaining chromatin was adjusted to the concentration of 1–2 million cells/ 100µl with IP dilution buffer (20mM Tris, 150mM NaCl, 2mM EDTA, 0.01% SDS, 1% Triton X-100 and 3 protease inhibitors). This lysate was distributed as 200-300µl / tube for optimum sonication. Sonication was performed using Bioruptor® Standard at H setting while alternating between 30s ON and OFF cycles for 10 minutes and repeating this 3 times. So the effective time per sample was 30 minutes. The ice inside the sonicator was changed after every 10 minutes to avoid heat degradation of the chromatin. After re-combining all the sonicated tubes, ~10µl of the chromatin was saved to confirm that the predominant size after sonication was ~500bp. In the meantime, Protein A/G Sepharose beads were washed and resuspended in IP dilution buffer (50µl/ Ab/ sample).

After sonication, the chromatin was centrifuged at 10000g at 4°C to save the supernatant. The supernatant was diluted using IP dilution buffer, such that there was 1ml chromatin/ Ab (KLF2 and Pre-immune serum) and 10% of the volume was saved as input (100µl). Rabbit raised anti-KL2 antibody (epitope: 90-255 amino acids of mouse KLF2) was prepared by Dr. Yousef Alhashem in the lab using previously described construct (Jiang et al., 2008). Approximately 3µg of KLF2 Ab, from aliquot 6, or pre-immune serum control D
were added to 1ml chromatin and 50µl of washed protein A/G sepharose affinity beads were added to facilitate precipitation. The chromatin was incubated overnight at 4°C on a rocker. The next day, beads were centrifuged out and washed sequentially twice with IP wash buffer 1 (20mM Tris, 50mM NaCl, 2mM EDTA, 0.1% SDS, 1% Triton X-100, pH 8.1) and once with IP wash buffer 2 (10mM Tris, 0.25M LiCl, 1mM EDTA, 1% Nonidet P-40, 1% desoxycholate, pH 8.1). Freshly prepared IP elution buffer (0.1M NaHCO₃, 1%SDS diluted to required concentration with water) was used to elute the chromatin IP. NaCl and Proteinase K were used to reverse-crosslinks at 65°C for ~6 hours. DNA was extracted, purified, precipitated and used for qPCR. Primers specific to promoter regions, of Tbx5, Gata4, UGDH and Sox9, with consensus KLF2 binding site were designed and are indicated in Table 4-2. Fold enrichment was calculated as \(2^{(C_{\text{input}} - C_{\text{test}})}\) and expressed relative to the preimmune serum control.

<table>
<thead>
<tr>
<th>Gene Promoter</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tbx5</td>
<td>GGAGACAGAAATCGGGTGAG</td>
<td>TTGCAGGGAGAAAGAAAAA</td>
</tr>
<tr>
<td>Gata4-100</td>
<td>AAACACGATCCTTGGCAGAG</td>
<td>GACTGGCCTAAGGGAGTCAC</td>
</tr>
<tr>
<td>Gata4-411</td>
<td>CCTTAAGGGCCAGTCAGGT</td>
<td>CTCTGCCAAGGATCGGTCTT</td>
</tr>
<tr>
<td>Sox9</td>
<td>CACACACACACATCGGTCA</td>
<td>AGACAGGAGGGAGGAAGAAG</td>
</tr>
<tr>
<td>UGDH</td>
<td>AGCACAGACAAGGATGACCA</td>
<td>GGCAGGCCTCTATTCTTCTC</td>
</tr>
</tbody>
</table>
Results

Candidate cardiovascular genes: Potential KLF2 targets

The candidate cardiovascular genes were selected based on criteria such as: literature to prove their role in AV cushion development, expression of these genes by E9.5 because this is when the phenotype is observed, and presence of the KLF2 binding consensus sequence (CCACCC and CCGCCC) (33) in the promoter of the gene. Table 4-3 shows the list of candidate genes that was tested for differential expression in FVB/N WT and KLF2-/- and mix WT and KLF2-/-.

Table 4-3: Candidate KLF2 targets in cardiovascular development

<table>
<thead>
<tr>
<th>Gene</th>
<th>Embryonic day and location of expression</th>
<th>KO phenotype</th>
<th>Presence of KLF2 binding consensus sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bmp2</td>
<td>E9.25 onwards Endo, Myo</td>
<td>Bmp2 knockout results in absence of cardiac jelly and consequently AV canal EMT.</td>
<td>1X in -500bp</td>
</tr>
<tr>
<td>Msx1 and Msx2</td>
<td>E9.5 day Endo, Myo</td>
<td>Absence of EMT. Hypocellular endocardial cushions.</td>
<td>2X in -250bp</td>
</tr>
<tr>
<td>Sox9</td>
<td>E9.5 onwards Endo, Mes</td>
<td>Endocardial cell migration is absent. Immature extracellular matrix.</td>
<td>3X in -250bp</td>
</tr>
<tr>
<td>Has2</td>
<td>E8.5 Endo, Myo, Mes</td>
<td>No endocardial cushion swelling. Alcian blue negative.</td>
<td>1X in -250bp, 2X in -750bp</td>
</tr>
<tr>
<td>Hey1/Hesr 1 and Hey2/Hesr 2</td>
<td>E9.0 onwards Endo, Myo</td>
<td>Reduced ventricular wall trabeculation, hypocellular AV cushions absence of EMT.</td>
<td>Hey1: 6X in -250bp, Hey2: 3X in -250bp</td>
</tr>
<tr>
<td>Gata4</td>
<td>E7.0 onwards Endo, Myo</td>
<td>Reduced ventricular wall trabeculation, VSD and ASD.</td>
<td>4X in -500bp</td>
</tr>
<tr>
<td>Tbx5</td>
<td>E9.0 onwards Endo</td>
<td>Hypoplastic endocardial cushion and ASD.</td>
<td>2X in -500bp</td>
</tr>
<tr>
<td>eNOS</td>
<td>E9.0 onwards Endo</td>
<td>Atrial Septal Defect</td>
<td>2X in -500bp</td>
</tr>
<tr>
<td>Notch1</td>
<td>E9.5 day Endo, Myo</td>
<td>Hypocellular endocardial cushions</td>
<td>2X in -250bp</td>
</tr>
<tr>
<td>Tgfβ2</td>
<td>E8.0 onwards Endo, Myo</td>
<td>Hypoplastic AV cushion development, myocardial defect</td>
<td>1X in -500bp</td>
</tr>
<tr>
<td>UGDH</td>
<td>E6.5 onwards Endo</td>
<td>No glycosaminoglycan synthesis. KO die by E8.0</td>
<td>1X in -500bp</td>
</tr>
</tbody>
</table>

Endo: Endocardium; Myo: Myocardium; Mes: Mesenchymal cells.
Dysregulation of cardiovascular genes in E10.5 FVB/N KLF2−/− whole hearts

To begin to elucidate the molecular mechanism by which KLF2 ablation results in heart development and septation abnormalities, the amounts of expression of candidate genes important in AV cushion development (Tbx5, Sox9, Tgfβ2, Notch1), atrial septation (Tbx5, Gata4) and glycosaminoglycan synthesis (UDP-glucose dehydrogenase or UGDH, and hyaluronase synthase 2 or Has2) were quantified in WT and KLF2−/− hearts. Each of these genes has at least one consensus KLF2 binding site in its promoter (CCACCC and CCGCCC) (Jiang et al., 2008), within 500 bp upstream of the transcription start site (Table 4-3).

The mRNAs for two cardiovascular transcription factors, Tbx5 and GATA4, showed significantly reduced expression in FVB/N KLF2−/− compared to FVB/N WT hearts (Figs. 4-2A and B), indicating that these genes are, directly or indirectly, positively regulated by KLF2. UGDH mRNA expression is also reduced in the absence of KLF2 (Fig. 4-2C) in FVB/N but not mixed background embryos.

The Notch1 (Fig. 4-2E), Tgfβ2 (Fig. 4-2G) and Sox9 (Fig. 4-2F) genes are important for atrioventricular development, but these mRNAs are not expressed significantly differently in KLF2−/− and WT hearts, whether the animals are in an FVB/N or a mixed genetic background (Fig. 4-2E, 4-2F and 4-2G). The data suggest that Tbx5, GATA4 and UGDH are downstream of KLF2 and have a genetic background specific role in AV endocardial cushion EMT, atrial septation and cardiac jelly synthesis. However whole hearts were used to extract mRNA thus the results were not specific enough to study dysregulation of these genes in the AV region. This might also show a more profound effect for some genes, since a large difference in the AVC might be masked by the same level of expression in the rest of the heart.
Figure 4-2: KLF2 regulates cardiovascular genes. Genes tested are important for AV cushion development (Tbx5, Sox9, TGFβ and Notch1), atrial septation (Tbx5, Gata4) and extracellular matrix synthesis (UGDH, Has2). (A – C) In E10.5 FVB/N KLF2-/- hearts there is decreased expression of (A) Tbx5 (p = 0.0175), (B) GATA4 (p = 0.0164) and (C) UGDH (p = 0.0204) mRNA compared to FVB/N WT, but MixWT and MixKLF2-/- hearts have no significant differences in expression of these genes. The expression of other cardiovascular genes such as (D) Has2, (E) Sox9, (F) Notch1, (G) TGFβ2 and (H) eNOS does not appear to be regulated by KLF2 in either genetic background. Error bars indicate standard deviation. N = 5
Important cardiovascular genes are downregulated in FVB/N KLF2-/- compared to WT AV regions

To study expression patterns of the candidate genes specifically in the AV region, qRT-PCR was performed using the microdissected AV region rather than whole hearts. The AV region was dissected as previously described in Chapter 2.

The expression of the candidate genes in E10.5 WT and KLF2-/- whole hearts was compared using qRT-PCR. Gata4, Tbx5 and UGDH mRNA showed about 2 fold decrease in expression in FVB/N KLF2-/- compared to FVB/N WT. None of the other genes showed any statistically different expression patterns in the absence of KLF2. Mix KLF2-/- whole hearts did not show differential expression of any of the candidate genes compared to mix WT (Fig. 4-3). However most of these genes are expressed only in the AV endocardial cushion region and using whole hearts to extract mRNA decreased specificity of the assay. Thus we decided to use AV regions to extract mRNA. It was anticipated that this might show a more profound effect for some genes, because large differences in the AVC might be masked by the same level of expression in the rest of the heart.

In the AV cushion region, the mRNAs for four cardiovascular transcription factors, Tbx5, Gata4, UGDH and Sox 9, showed significantly reduced expression in FVB/N KLF2-/- compared to WT (Figs. 4-3A-4-3D), indicating that these genes are, directly or indirectly, positively regulated by KLF2. Other roles of Gata4 and Tbx5 are discussed in detail later, but Gata4 and Tbx5 double heterozygous knockout mice show myocardial thinning (Garg et al., 2003; Maitra et al., 2009), like KLF2-/- MixKLF2-/- has myocardial thinning but no reduction of Gata4 and Tbx5 mRNA, indicating that an additional unknown gene(s) is related to this phenotype. Sox9 plays an important role during endocardial cushion EMT as well as valve remodeling (Lincoln et al., 2007). As shown in Fig. 4-3 the expression of UDP-glucose
dehydrogenase (UGDH) mRNA was significantly reduced in FVB/N KLF2-/- compared to FVB/N WT, but similar amounts were expressed in mixWT and mixKLF2-/- (Fig. 4-3D). This correlates with the reduced alcian blue staining in FVB/N KLF2-/-, but not in mixKLF2-/-, compared to WT. Expression of the hyaluronan synthase 2 (Has2) gene was not reduced in FVB/N KLF2-/- compared to WT AV canals (Fig. 4-3A). Therefore a reduction in Has2 is not likely to be the cause of the reduced GAGs observed in FVB/N KLF2-/- hearts.

The Has2, Notch1, Tgfβ2 and eNOS genes are important for atrioventricular development, but these mRNAs are not expressed significantly differently in KLF2-/- and WT hearts, whether the animals are in an FVB/N or a mixed genetic background (Fig. 4-4A – 4-4C).
Figure 4-3: Cardiovascular genes are dysregulated in FVB/N KLF2-/- AV region. Quantitative RT-PCR (qRT-PCR) of AV region RNA was used to test the amount of expression of genes important for AV cushion development (Tbx5 and Sox9) and atrial septation (Tbx5 and Gata4). Cyclophilin A mRNA was used as a normalization control. FVB/N WT and mixWT were set to 100%, and KLF2-/- were compared to WT. (A – C) In E10.5 FVB/N KLF2-/- AV region there is decreased expression of (A) Tbx5 (p = 0.0175), (B) Gata4 (p = 0.0164) and (C) Sox9 (p = 0.019) mRNA compared to FVB/N WT, but MixWT and MixKLF2-/- hearts have no significant differences in expression of these genes. Error bars indicate standard deviation. D) qRT-PCR shows 2-fold decrease in expression of UGDH mRNA (p = 0.0204) in FVB/N KLF2-/- but not mixKLF2-/- compared to WT AV region. n = 5.
Figure 4-4: Cardiovascular genes that are not differentially expressed in WT and KLF2−/− AV region. Genes tested are important for extracellular matrix synthesis (Has2) and AV cushion development (TGFβ2 and Notch1). AV region RNA was used in qRT-PCR. There was no significant difference in expression of A) Has2; B) Notch1 and C) TGFβ2 in KLF2−/− compared to WT AV regions. n= 5
Expression profiles of cardiac genes in endothelial specific KLF2-/- AV canals

E10.5 endothelial-specific Tie2-cre KLF2-/- (Tie2-cre KLF2-/-) hearts show hypocellular endocardial cushions, disorganized AV canal regions, and delayed atrial septal formation (Fig. 2-4G and 2-4H), similar to the traditional KLF2 KO. Using this model, the deletion of the KLF2 gene in the heart is quite complete at approximately 84% (unpublished data). Negative control littermates having the floxed KLF2 gene without Tie2-cre are normal. This suggests that KLF2 has an endothelial cell-autonomous role in the AV cushion region. To determine if the same genes are differentially expressed when KLF2 is absent only from endothelial cells in the AV region, quantitative RT-PCR was performed on conditional KLF2-/- FVB/N Tie2-cre KLF2-/- AV canals show a similar and significant reduction in the expression of the Tbx5, Gata4, UGDH and Sox9 genes, compared to controls without Tie2-cre (Figure 4-5).
Figure 4-5: Tie2-cre KLF2-/- AV regions have decreased expression of cardiovascular genes, similar to the traditional KLF2 KO. Quantitative RT-PCR (qRT-PCR) of AV region RNA was used to test the amount of expression of genes important for AV cushion development (Tbx5 and Sox9), cardiac jelly synthesis (UGDH) and atrial septation (Tbx5 and Gata4). Cyclophilin A mRNA was used as a normalization control. WT (KLF2<sup>fl/fl</sup>, without Tie2-cre) were set to 100% and Tie2-cre KLF2-/- were compared to them. In E10.5 Tie2-cre KLF2-/- AV region there is decreased expression of Gata4 (p = 0.0072), Tbx5 (p = 0.006), UGDH (p = 0.0012) and Sox9 (p = 0.0014) mRNA compared to WT. All animals are in FVB/N background. Error bars indicate standard deviation. Student’s t-test was used to compare WT and KLF2-/- gene expression. The brackets indicate significant differences in mRNA expression between WT and KLF2-/-, n = 6.
**KLF2 binds to the mouse Gata4, Tbx5 and UGDH promoters**

KLF2 positively regulates mRNA expression of Gata4, Tbx5, Sox9 and UGDH. To better understand the mechanism of KLF2 regulation, chromatin immunoprecipitation (ChIP) assays using a KLF2 polyclonal antibody (Jiang et al., 2008) were performed using cells from E10.5 WT mouse AV regions. Due to the limited availability of tissue per sample, between eight and ten E10.5 AV regions were pooled for each ChIP assay. The Gata4, Tbx5 and Sox9 promoters have multiple potential KLF2 binding sites, and the UGDH promoter has a single site (Table 4-4). Due to assay limitations, the two Tbx5 and two Sox9 binding sites could not be distinguished from each other, and were tested simultaneously. Two regions of the Gata4 promoter, each containing two putative KLF2 binding sites (designated -100 and -411), were tested for KLF2 enrichment. The data in Figure 4-6 indicates that KLF2 showed an approximately 70-fold enrichment at the Tbx5 promoter, 25-fold enrichment at the UGDH promoter, and 15-fold enrichment at the proximal Gata4 promoter site (-100) compared to negative control assays using pre-immune serum. No significant KLF2 enrichment was observed at the Sox9 promoter. As a negative control, KLF2 does not bind to the promoter of the β-actin gene. The ChIP assays thus indicate that KLF2 binds to the Gata4, Tbx5 and UGDH promoters, as would be expected if it directly regulates these genes. No evidence was obtained to indicate that KLF2 directly regulates the Sox9 gene.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Location of KLF2 consensus binding site with respect to transcription start</th>
<th>KLF2 binding site sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tbx5</td>
<td>-389, -333</td>
<td>CCGCCC, CCACCC</td>
</tr>
<tr>
<td>Gata4</td>
<td>-411, -338, -85, -78</td>
<td>CCACCC, CCGCCC</td>
</tr>
<tr>
<td>Sox9</td>
<td>-172, -250</td>
<td>CCACCC</td>
</tr>
<tr>
<td>UGDH</td>
<td>-73</td>
<td>CCACCC</td>
</tr>
</tbody>
</table>
Figure 4-6: ChIP assays were performed on cells obtained from E10.5 WT AV canal and cushion regions. Approximately 8 to 10 WT AV regions were pooled to obtain cells for one replicate. Polyclonal anti-KLF2 and non-specific control pre-immune serum was used. The y-axis represents the relative fold-enrichment. The mean pre-immune enrichment was set as ‘1.0’ and the enrichment with KLF2 antisera was scaled appropriately. The x-axis indicates the location of the primers used for qPCR; all were in gene promoters and are described in Supplementary Table 3. Pr: Promoter. Primers specific for the β-actin gene were used as a negative control. n = 7 biological replicates.
Discussion

In this work, we have demonstrated that KLF2 binds the promoters of, and positively regulates, the Tbx5, Gata4 and Ugdh genes in the mouse E10.5 AV canal. An endocardial specific Gata4 KO has multiple layers of endocardium in the AV canal, and hypocellular AV cushions at E10.5 (Rivera-Feliciano et al., 2006), similar to the FVB/N KLF2-/-. Tbx5 KO embryos have hypoplastic endocardial cushions (Bruneau et al., 2001), like FVB/N KLF2-. These phenotypes correlate with our observation that Gata4 and Tbx5 expression is reduced approximately 3-fold in the absence of KLF2 in FVB/N AV canals. Interestingly, the Tbx5 and Gata4 proteins physically interact during cardiac development. A heterozygous mutation (mG295S) in the Gata4 gene disrupts these protein interactions, resulting in cardiac defects like atrial septal defects (ASD), AV septal defects (AVSD) and myocardial thinning beginning at E11.5 (Garg et al., 2003; Maitra et al., 2009). The AVSD and ASD in these mice are known to result from abnormal EMT and remodeling of endocardial cushions. Gata4 mG295S is a missense mutation resulting in diminished DNA binding affinity and transcriptional activity, making it similar to a null allele. In mice with this Gata4 mutation and a null allele for Tbx5, Gata4+/-Tbx5+/-, there is normal EMT but defective remodeling, resulting in septal defects (Maitra et al., 2009). Like these double heterozygotes, FVB/N KLF2-/ embryos have about 50% less Gata4 and Tbx5 mRNA than normal, and similarly remodeling is affected. However, EMT is affected, indicating that the KLF2-/- embryos are more severely affected than Gata4+/-Tbx5+/-, likely because KLF2 also controls other cardiac genes.

KLF2 binds to and positively regulates the UDP-glucose dehydrogenase (UGDH) gene. UGDH is expressed in the endocardium and catalyzes conversion of UDP-Glucose to UDP-Glucuronic acid (UDP-GA) (Walsh & Stainier, 2001). UDP-GA is further converted to hyaluronic acid and other glycosaminoglycans by hyaluronan synthase 2 (Has2) (Joziass et
There is an approximately 2-fold reduction in UGDH mRNA in the FVB/N KLF2-/- AV canal. This may result in decreased production of UDP-GA and consequently reduced glycosaminoglycans in the cardiac jelly. Interestingly, two missense mutations in the UGDH gene were recently identified in 3 patients with congenital valve defects (Hyde et al., 2012; Smith et al., 2009). These mutations result in structural defects in UGDH that significantly compromise enzyme function (Hyde et al., 2012). These findings support our hypothesis that reduced UGDH contributes to the cushion defects in KLF2-/- mice.

Sox9 is a cardiovascular transcription factor expressed in endothelial and mesenchymal cells in the endocardial cushion region (Lincoln et al., 2007). In our study, the Sox9 gene is positively controlled by KLF2, but the lack of evidence for KLF2 promoter binding suggests that the regulation is indirect, or controlled by a more distant DNA element. Sox9 KO results in hypoplastic endocardial cushions and abnormal valve formation (Akiyama et al., 2004). A study by Lincoln et al. showed that ablation of Sox9 in endocardial cells results in reduced EMT (Lincoln et al., 2007). These phenotypes are similar to FVB/N KLF2-/- hearts. The reduced expression of Sox9 in KLF2-/- hearts could be attributed to a reduced number of mesenchymal cells in the AV cushion. However, this seems unlikely because Has2 mRNA, which is also expressed in endocardial and mesenchymal cells, does not show reduced expression in KLF2-/- AV regions. Sox9 mRNA showed reduced expression in AV region but not whole heart, indicating that in absence of KLF2, Sox9 is highly dysregulated in the AV region.

This study thus clearly suggests downstream targets of KLF2 in cardiac development. KLF2 does not seem to be part of any particular signaling pathway summarized in Fig. 4-1. But it regulates other transcription factors that are common components of all of the involved pathways. A simple representation of the KLF2 action is shown in Fig. 4-7. Further study would be needed to determine which of the KLF2 promoter
binding sites discovered are responsible for direct regulation of genes by KLF2. Luciferase assays could be performed by co-transfecting KLF2 expression and promoter-luciferase gene constructs into HUVEC cells.

The method used to select the candidate genes is somewhat biased. We could have missed potential KLF2 target genes that are not only instrumental in heart development, but also play other important roles earlier in embryogenesis. The literature on such genes would thus not mention a heart defect if the embryos die before heart development begins. Thus to exhaustively identify KLF2 target genes, gene expression microarrays and ChIP-seq could be used.

![Diagram](image.png)

**Figure 4-7: Cardiac genes directly and indirectly regulated by KLF2.** Solid single-headed arrow indicates direct regulation by KLF2. Dotted arrow indicates that KLF2 positively regulates Sox9, but there is no evidence of KLF2 binding the Sox9 promoter. The double headed arrow between Gata4 and Tbx5 represents the interaction between these proteins during development.
CHAPTER 5: KLF2 AND KLF4 PLAY COORDINATE ROLES IN MAINTAINING VASCULAR INTEGRITY DURING MOUSE DEVELOPMENT

Summary

During embryonic development, KLF2 plays an important role in vessel maturation. In adult mice KLF2 regulates expression of the tight junction protein occludin, which may allow KLF2 to maintain vascular integrity. Adult conditional KLF4 knockout mouse embryos have thickened arterial intima following vascular injury (Yoshida, Gan, & Owens, 2008). However the role of KLF4 in developing vasculature is not well studied (Lin et al., 2010). Breeding KLF2+/− and KLF4+/− mice resulted in the generation of KLF2/KLF4 double knockout embryos. KLF2−/−KLF4−/− embryos in an undefined mixed genetic background die before E10.5 and often show cranial bleeding. This is earlier than either single knockout. Light microscopy was used to study the cardiovascular structural phenotypes in E10.5 FVB/N KLF2−/− and E9.5 KLF2−/−KLF4−/− embryos. E10.5 FVB/N KLF2−/− show gaps in the endothelial lining at the dorsal aorta and a number of blood cells localized outside the aorta suggesting either hemorrhaging or inability of the hematopoietic progenitors to reach the aortic endothelium and enter circulation. Microscopy confirmed hemorrhaging near and endothelial breaks in the primary head vein (PHV) in E9.5 KLF2−/−KLF4−/− (n=3) and E10.5 KLF2−/−KLF4+/− embryos (n=1). The role of KLF2 and KLF4 in vascular development has not been studied as much as adult vascular regulation. This study begins to define the roles of these two transcription factors in the development of blood vessels.
Introduction

Endothelial precursors or angioblasts initiate intraembryonic vascular development by E7.0. During vasculogenesis, angioblasts form a primary capillary plexus (Reviewed in (Rossant & Howard, 2002)). In mice and humans, the vasculature is further extended by sprouting and remodeling of these plexuses, a process called angiogenesis that continues from early embryogenesis to adulthood. Ephrin/Eph bidirectional signaling and Notch signaling play important roles in artery and vein formation and specification (Reviewed in (Rossant & Hirashima, 2003)). During maturation, endothelial cells recruit mesenchymal cells or vascular smooth muscle cell (VSMC) progenitors to the surface of the vessels, and these cells organize into layers around the tube to support it (Reviewed in (Cleaver & Melton, 2003)). Transforming Growth Factor-β (TGF-β) is important in differentiation of mesenchymal cells to form VSMCs and phenotypic switching of VSMCs (Adam, Regan, Hautmann, & Owens, 2000). Endothelial cells also promote VSMC differentiation and maintain the differentiated phenotype from E7.5 onwards (Drake et al., 2000).

KLF2 in vascular development

KLF2 has been extensively studied in vascular endothelial biology. The gene is expressed in endothelial cells in the developing mouse embryo as early as E8.5 (Lee et al., 2006). KLF2 knockout mouse embryos die in utero between E11.5-E14.5 (Kuo, Veselits et al., 1997; Lee et al., 2006; Wani et al., 1998). Angiogenesis and vasculogenesis appear grossly normal at E11.5. Kuo et al. examined KLF2 traditional knockout embryos, and concluded that death is due to hemorrhaging and a lack of integrity in smooth muscle layers surrounding vessels from around E11.5 (Kuo, Veselits et al., 1997). Hemorrhaging at the same developmental stage was also detected in KLF2-/- embryos developed by another group (Wani et al., 1998). Kuo et al. found that KLF2 is required for normal development of
tunica media surrounding the large vessels of the embryo, including the aortae, and umbilical veins and arteries (Kuo, Veselits et al., 1997).

In another study, Wu et al. showed that traditional KLF2-/- embryos have normal endothelial cell development, but a failure of mural cells to migrate around endothelial cells to stabilize the blood vessels. Platelet-derived growth factor (PDGF) is known to regulate VSMC migration. Using KLF2-/- mouse embryonic fibroblasts in culture, the authors showed that KLF2 is involved in PDGF-β induced migration (Wu et al., 2008).

Lee et al. examined KLF2 endothelial-conditional knockout embryos, and concluded that they ultimately die from heart failure, due to reduced smooth muscle tone causing high cardiac output (Lee et al., 2006). In contrast to the previous studies, the authors observed no hemorrhaging, or vascular smooth muscle abnormalities in any of the great vessels, in Tie2-Cre KLF2 knockout embryos. It was previously reported that traditional KLF2 knockout embryos had normal cardiac development at E12.5, but Lee et al. noted that in E11.5 KLF2 endothelial-conditional knockout embryos, the myocardial cell layers in the heart appear to be thinner than in wildtype (Lee et al., 2006). A common theme drawing all of these studies together is that endothelial expression of KLF2 is required for VSMC function in nascent vessels. A line of reasoning which is not shared by Lee et al., but is supported by the other studies, is that KLF2 expression is required for VSMC recruitment. The experiments of Lee et al. differ from the others in that KLF2 is ablated only in endothelial cells. It is possible that VSMC recruitment is negatively affected in these other studies due to the absence of KLF2 expression in a cell type other than endothelial cells, although KLF2 expression in VSMC has not been demonstrated. The genetic background of the mice often influences the cardiovascular phenotype. The mice used in all of the above studies were in an undefined genetic background. This could be one reason for variable vascular phenotypes in these studies.
In tissue culture, KLF2 plays a role as a molecular transducer of fluid shear forces, thus directly or indirectly regulating a number of endothelial genes (Dekker et al., 2005). Recent findings suggest that KLF2 plays an important role in endothelial barrier function in adult mice. Using KLF2+/− mice under vascular stress, they showed that KLF positively regulates expression of the tight junction protein occludin and modification of myosin light chain that is important for the integrity of the endothelial layer and to avoid vascular leakage (Lin et al., 2010). In HUVECs exposed to a biomechanical stimulus characteristic of atheroprotected regions, KLF2 positively regulates the expression of vasoprotective genes and inhibits expression of pro-inflammatory genes in endothelial cells and macrophages, preventing atherosclerosis (Parmar et al., 2006).

**KLF4 in vascular development**

KLF4 is another member of the Krüppel-like transcription factor family. It is expressed in extraembryonic tissue from E4.5 onwards and in the mesenchymal tissue and epithelium by E10.5 (Ehlermann, Pfisterer, & Schorle, 2003). KLF4 is important in skin barrier function during development (Segre, Bauer, & Fuchs, 1999), and in the phenotypic switching of vascular smooth muscle cells (Liu et al., 2005). KLF4 knockout mice die soon after birth due failure of the skin barrier function. However, no vascular abnormality has been reported during their embryonic development (Segre et al., 1999). KLF4 mRNA has been shown to be expressed in endothelial cells of adult C57/BL6 mice, in both arteries and veins by northern blot and in situ hybridization.

KLF4 is induced by laminar shear stress in endothelial cells (HUVECs) and it further induces expression of endothelial NOS (eNOS) and thrombomodulin (TM) by transactivating the two promoters. TM and eNOS are important in vascular tone regulation and maintenance of intact endothelium (Yoshida et al., 2008). KLF4 expression in VSMCs under basal
conditions is very low. Thus in normal vascular smooth muscle cells (VSMCs), KLF4 is not responsible for repression of SMC differentiation marker genes like α- smooth muscle actin (α-SMA) and SM22α. Under conditions of vascular stress, however, KLF4 is upregulated and represses SMC genes by both down-regulating myocardin expression and preventing myocardin from associating with SMC gene promoters in cultured VSMCs. In vascular injury in adult mice, KLF4 is upregulated and plays a role in phenotypic switching by down-regulation of multiple SMC marker genes (Kawai-Kowase & Owens, 2007).

In adults, KLF4 regulates the inflammatory response of the endothelium. Like shear stress, pro-inflammatory mediators like TNFα, IL-1β or IFNγ induce endothelial KLF4 expression. KLF4 inhibits expression of inflammatory mediators such as TF and VCAM-1. It might also promote blood fluidity and prolong clotting times, since it upregulates TM. Thus it is responsible for anti-inflammatory, anti-coagulant state in endothelial cells (Hamik et al., 2007).

The potential roles of KLF4 during vascular development in mice are less explored. Its role in vascular regulation has been studied either in vitro or in adult mouse models. Thus it will be interesting to observe the vascular phenotype of KLF4−/− embryos.

Role of KLF2 and KLF4 in vascular development

MEK5/ MEF2-dependent signaling pathway induces expression of KLF2 and KLF4 in response to vascular stress. Genome-wide transcriptional profiling of endothelial cells (HUVECs) overexpressing KLF4, KLF2, or constitutively active MEK5 revealed that 59.2% of the genes regulated by the activation of MEK5 were similarly controlled by either KLF2 or KLF4. This suggests similar and complementary roles of KLF2 and KLF4 in vasoprotection (Villarreal et al., 2010). There is structural homology in KLF2 and KLF4 and the fact that both
have similar roles in vascular regulation suggests that they might have overlapping roles in
development as well. We thus hypothesize that KLF2 and KLF4 might have compensatory
roles in maintaining integrity during blood vessel development. The preliminary results in our
lab support this hypothesis. Previous work in the laboratory shows that KLF2/KLF4 double
mutant embryos die by E10.5, which is sooner than the single mutants of either KLF2 or
KLF4. KLF2-/-KLF4-/- embryos but neither single mutant shows gross hemorrhaging and
enlarged blood vessels in the head at E10.5 (Figure 5-1). At E10.5, KLF2-/-KLF4+/- embryos
display diminished recruitment of mesenchymal cells at the primary head vein in the cranial
cross-section, thus affecting maturation of blood vessels. The endothelial cell lining of the
primary head vein and the carotid artery is not continuous indicating a lack of vessel
integrity. These findings are not observed in single mutants (Figure 5-2). KLF2-/-KLF4+/-
embryos were used because KLF2/KLF4 double knockout embryos disintegrate by the time
they are embedded in plastic and thus sections cannot be observed by light microscope.
KLF2-/- KLF4-/- embryos at E9.5 will be observed for hemorrhaging and vascular disintegrity
phenotypes because they are dead at E10.5.
Figure 5-1: Ablation of KLF2 and KLF4 leads to hemorrhaging. Light micrographs of E10.5 mouse embryo whole mounts were taken at 16X magnification. A) WT; B) KLF4-/-; C) KLF2-/- embryos are grossly normal. D) KLF2+/-KLF4-/-; E) KLF2-/-KLF4+/-; F) KLF2-/-KLF4-/- embryos show hemorrhaging and/or enlarged blood vessels in the head. EV: Enlarged blood vessels; H: Hemorrhaging. n = 3. This experiment was performed by a graduate student in the lab, Sean Fox.
Figure 5-2: Double KO Primary head vein lacks continuous endothelial layer and have reduced numbers of mesenchymal cells near primary head vein. Light micrographs of 7µ sections were taken at 200X magnification. A) WT shows normal looking primary head vein; B) In KLF2+/−/KLF4−/− the endothelial layer is apparently continuous, but have less number of mesenchymal cells around the primary head vein; C) KLF2−/−/KLF4+−/− shows apparently continuous endothelial layer, less mesenchymal cells and blood cells outside the primary head vein; D) KLF2−/−/KLF4−/− primary head vein lacks continuous endothelial layer, lesser mesenchymal cells and blood cells outside the vessel. EL: Endothelial layer; MC: Mesenchymal cells; B: Blood cells. This experiment was performed by Sean Fox.
Methods

Generation and Genotyping of mice

KLF2+/ mice were generated by and obtained from Dr. Jerry Lingrel (Wani, 1998). KLF4+/ mice were generated by and obtained from Dr. Julie Segre (Segre, 1999). KLF2 and KLF4 are located on mouse chromosomes 8 and 4, respectively, and therefore segregate independently in meiosis. KLF2+/ and KLF4+/ mice were bred to generate KLF2+-KLF4+/- double heterozygous mice. KLF2+-KLF4+/- males were mated with wild type (WT) FVB/N females to produce additional KLF2+-KLF4+/- mice to preserve the line. KLF2+-KLF4+/- were bred with KLF2+-KLF4+/- males. This mating generated homozygous KLF2--KLF4+-/- double knockout (DKO) embryos and embryos to serve as controls: WT, KLF2+-KLF4+, KLF2--KLF4-, KLF2+-KLF4-, KLF2+-KLF4+-, KLF2--KLF4--. The offspring of this matings have an undefined mixed genetic background including FVB/N, C57BL/6 and 129/Sv. Presence or absence of KLF2 was detected by PCR using primers described in Chapter 2. KLF4 allele was detected by PCR using following primers. Forward normal primer: 5’ CAGCTTCATCCTCGTCTTCC 3’; Reverse normal primer: 5’ AGACGCCTTCAGCACAACGT 3’; Forward knockout primer: 5’ GCCAGTCATAGCCGAATAG 3’; Reverse knockout primer: 5’ GACCAGAATAGAGTCAAGGGTTAGG 3’. The KLF4 WT band size using above primers is 320bp and KLF4 KO band size is 270bp.

E9.5 dissection, crown to rump length measurement and sample preparation

Dissection, plastic embedding, sectioning, staining and light microscopy techniques for E9.5 embryos were similar to those described in Chapter 2 for E10.5 embryos. The only difference was that the embryos were earlier in gestation, and therefore smaller in size. The embryo was detached and and photographed on a black background with an Olympus Q-Color 3 camera using QCapture 2.81.0 imaging software, after aligning the embryonic spine
with a metric ruler. The length was measured from the tip of the head of the embryo to the rump as shown in Fig. 5-3.

![Image](image_url)

Figure 5-3: Crown to rump length. E9.5 whole embryo placed next to the metric scale. The horizontal black lines on the head and rump of the embryo indicate crown and rump respectively.

**Benzidine staining**

Benzidine staining was performed on 4% PFA fixed cryosections of FVB/N WT and KLF2-/- embryos. Sections showing descending aorta were selected and submerged in 1X PBS for 1 hour before the benzidine staining process. PBS washes removes OCT, cryo-freezing medium. This step is required to remove OCT cryo-freezing medium. This critical step was advised in the protocol kindly provided by Dr. Terry Magnuson’s lab. Sections were then incubated in methanol for 15 seconds. Methanol improves permeability of the tissue. The sections were stained with ~100µl of 1% 3, 3’, 5, 5’ Tetramethylbenzidine (TMB) in methanol for 5 minutes, 2.5% hydrogen peroxide (H₂O₂) in 70% ethanol for 3 minutes and washed with DI water for 2.5 minutes. The heme group in the red blood cells catalyzes the breakdown of H₂O₂. As H₂O₂ reduces to water, TMB is oxidized to TMB diamine imparting a golden brown color in basic pH and a blue color in acidic pH (Griffin et al., 2008).
Results

*E10.5 FVB/N KLF2-/- embryos show a lack of vascular integrity at the dorsal aorta*

E10.5 FVB/N KLF2-/- embryos display abnormal cardiac function. Normal vascular and cardiac development contributes to optimal heart function. Thus the major blood vessels in KLF2-/- were observed, using light microscopy and compared with WT (Fig. 5). Similar to the KLF2-/-KLF4+/- primary head vein (Fig. 5-2), FVB/N KLF2-/- embryos show erythroid cells outside of the dorsal aortas, suggesting possible hemorrhaging (Fig. 5-4B and 5-4C). Alternatively, the presence of erythroid cells outside of the FVB/N KLF2-/- dorsal aortas may be due to an inability of hematopoietic progenitors, originating in the para-aortic mesenchyme, to reach the aortic endothelium and enter circulation (Wood, May, Healy, Enver, & Morriss-Kay, 1997).

To confirm that these cells are erythroid, benzidine staining was performed. In FVB/N KLF2-/- embryos, there are benzidine-positive cells in the tissue surrounding the dorsal aorta (Fig. 5-4D), but they are found only within the dorsal aorta in FVB/N WT (Fig. 5-4E). The area outside the vessel also shows a lower density of mesenchymal cells compared to WT, indicating abnormal tunica media as described by Kuo *et al.* These results were replicated in E10.5 FVB/N WT and KLF2-/- hearts at the 34 and 36 somite stages. There are no blood cells in the tissues surrounding the dorsal aortae in mixKLF2-/- embryos (Fig. 5-4G and 5-4H), which look comparable to mixWT (Fig. 5-4F) and FVB/N WT (Fig. 5-4A).
Figure 5-4: E10.5 FVB/N KLF2-/- but not mixKLF2-/- embryos have erythroid cells outside of the dorsal aortas. (A) is a light micrograph (100X) of the FVB/N WT E10.5 dorsal aortas (DA). Erythroid cells (Ery) are found only within the aortas. (B) FVB/N KLF2-/- have a number of erythroid cells in tissue surrounding the DA. The box indicates the region that is magnified in C (200X). (C) Erythroid cells within and outside the vessel in FVB/N KLF2-/-. (D) and (E) are light micrographs of benzidine-stained WT and KLF2-/- sections, respectively (400X). Ery indicates benzidine-positive, brown-colored erythroid cells. Mes indicates mesenchymal cells. (F), (G) and (H) are mixWT (100X), mixKLF2-/- (100X) and mixKLF2-/- (200X), respectively, showing normal aortas with erythroid cells within the vasculature only. (n = 3 each).
**Gross development of E10.5 KLF2-/-KLF4-/-**

KLF2 and KLF4 individually play a role in vascular development. Previous light microscopy observations in our laboratory have shown that at E10.5, the simultaneous absence of KLF2 and KLF4 results in hemorrhaging indicating an apparent loss of endothelial integrity in the cranial region (Fig. 5-1). The expected number of E10.5 KLF2-/-KLF4-/- were obtained from mating KLF2+/KLF4+/ males and females (Table 5-1). However no heartbeat was observed in any of the 3 double knockout embryos, indicating that they were dead but not re-absorbed. To assess the gross development of the embryos, crown to rump length of E10.5 embryos of all the relevant, control and intermediate genotypes was obtained as shown (Fig. 5-5). The intermediate genotypes of this mating are: KLF4+-, KLF2+/-, KLF2+/-KLF4+/-, KLF4-/-, KLF2-/-, KLF2+/-KLF4-/- and KLF2-/-KLF4+/-.

The double knockout embryos observed are not statistically significantly smaller than any of the controls (Student’s t-test, p-value = 0.721). Thus there seems to be no apparent effect of the double knockout on the size of the embryo.

| Table 5-1 Genotypes of E10.5 embryos from KLF2+/-KLF4+/ matings |
|-----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Expected        | 4      | 8     | 8     | 16            | 4              | 4             | 8              | 8              | 4              |
| Observed        | 5      | 9     | 9     | 13            | 3              | 3             | 12             | 7(3)           | 0(3)           |
| Parentheses     |        |       |       |               |                |               |                |                |                |
| Indicator       |        |       |       |               |                |               |                |                |                |

This data was obtained by Sean Fox.
Figure 5-5: Bar graph representing crown to rump lengths of E10.5 embryos from KLF2+/-KLF4+/- matings. The Y axis represents crown to rump length in millimetres and the X axis represents various genotypes that are outcome of the KLF2+/-KLF4+/- mating. In parentheses is the sample size of every genotype observed. There was not statistical significance in the crown to rump length. Student’s t-test p-value= 0.721.
**E9.5 KLF2-/-KLF4-/- show hemorrhaging in the cranial region**

Light micrographs of E9.5 embryos of the following genotypes were obtained: WT (n = 4), KLF2+/-KLF4+/- (n = 7), KLF2-/- (n = 2), KLF4-/- (n = 1), KLF2-/-KLF4+/- (n = 4), KLF2+/-KLF4-/- (n = 3), and KLF2-/-KLF4-/- (n = 3). At E9.5, the expected numbers of all genotypes were obtained. Various endothelial abnormalities were observed in KLF2-/-KLF4-/- and KLF2-/-KLF4+/- embryos. One KLF2-/-KLF4-/- embryo shows gross hemorrhaging in whole-mount, in the cranial region, beginning rostrally at the level of optic vesicle and ending caudally at the level of the first branchial arch (Figure 5-6). In the whole mount as well as light micrographs, the hemorrhaging in this embryo was confined to the primary head vein (PHV) and the rostral portion of the anterior cardinal vein, into which the PHV drains. Bleeding penetrated at some levels, all the way to the midline, infiltrating the supporting cranial mesenchyme, a precursor tissue to vascular smooth muscle.

Figure 5-7 shows images of KLF2+/-KLF4+/- and KLF2-/- sections that indicate intact vasculature at the same level as the double knockout. While only one E9.5 KLF2/KLF4 DKO embryo at this age (n=3) showed gross bleeding, all E9.5 KLF2/KLF4 DKO embryos showed endothelial disruption of the PHV at the level of the optic vesicle in transverse sections (Fig. 5-7), making this a common finding. All of the double knockout embryos had a heartbeat indicating that they were alive at E9.5. In these embryos, erythroid cells were observed outside the lumen of the PHV, with apparent gaps between joining endothelial cells. No obvious reduction in surrounding mesenchyme, as observed at E10.5 in Fig. 5-4B, was observed. Endothelial disruption of the PHV was also seen in two KLF2-/-KLF4+/- embryos (n=4) (Figure 5-7), as observed at E10.5. While erythroid cells were more difficult to identify in the E9.5 KLF2-/-KLF4+/- and double KO sections compared to E10.5 KLF2-/-KLF4+/- (Fig. 5-2), the presence of apparent gaps in the endothelial layer suggested vascular disintegration (Fig. 5-7). Benzidine staining should be performed to confirm the presence of the
blood cells outside of the vasculature. No abnormal phenotype was seen in KLF2+/−-KLF4−/− embryos, indicating that KLF2 ablation may be required for the abnormal vascular phenotype. These embryos were in undefined mixed background with ~50% FVB/N.

Figure 5-6: At E9.5 ablation of KLF2 and KLF4 leads to cranial hemorrhaging.
Light micrographs of E9.5 mouse embryo whole mounts were taken at 16X magnification. A) WT; B) KLF2−/−; C) KLF4−/−; D) KLF2+/−-KLF4−/−; E) KLF2−/−-KLF4+/− embryos are grossly normal. F) KLF2−/−-KLF4−/− embryo shows hemorrhaging and/or enlarged blood vessels in the head region. Arrowhead indicates hemorrhaging. n = 5.
Figure 5-7: Double KO Primary head vein lacks continuous endothelial layer Light micrographs of 7µ sections were taken at 200X magnification. A) WT shows normal looking primary head vein; B) KLF2-/-KLF4+/- shows gaps in the endothelial layer of the primary head vein; D) KLF2-/-KLF4-/- primary head vein lacks continuous endothelial layer, lesser mesenchymal cells and blood cells outside the vessel. Arrowheads indicate the gaps in the endothelial layer. PHV: Primary Head Vein
Discussion

KLF2-/KLF4-/ embryos in an undefined mixed genetic background apparently die by E10.5, which is earlier than the reported death of KLF2 KO embryos in undefined mixed background (E12.5 – 14.5) (Lee et al., 2006). KLF4 KO mice die postnatally.

The E9.5 KLF2-/KLF4-/ phenotype shows gross and microscopic hemorrhaging, also observed in E10.5 KLF2-/KLF4+/-, E10.5 KLF2-/KLF4-/-, and E9.5 KLF2-/KLF4+-/- mutants. The severity of cranial hemorrhaging increases from E10.5 KFL2-/KLF4+-/- to E10.5 KLF2-/KLF4--. This indicates that the loss of one additional KLF gene negatively impacts the vascular development of the mouse embryo. The effect of gene dosing is also seen in Hoxa-13 and Hoxd-13 mice (Warot, Fromental-Ramain, Fraulob, Chambon, & Dolle, 1997). The combined effect of deleting both genes is embryonic lethal. The mice of genotype Hoxa-13+/Hoxd-13-/- reach adulthood but with more severe abnormalities of the genitourinary and digestive systems than seen in Hoxa-13-/ mutants or Hoxa-13+/Hoxd-13+/ double heterozygotes.

Compared to KLF4, ablation of KLF2 disproportionately affects vascular development in the mouse embryo. KLF2 is important for vascular integrity (Lin et al., 2010). Kuo et al. (1997) and Wani et al. (1998) reported hemorrhaging in the abdominal and cardiac outflow tract region in KLF2-/ embryos (Kuo, Veselits et al., 1997; Wani et al., 1998). To the contrary, no hemorrhaging was observed in the KLF2-/ embryos examined by Lee et al. (2006) (Lee et al., 2006). This discrepancy may be partially explained by the current study. Erythroid cells were observed outside E10.5 KLF2-/ dorsal aortas in FVB/N but not in mixed genetic background embryos, indicating that this phenotype is genetic background-specific.
The genetic background of the KLF2-/ mice used in the previous studies was not well defined. Likewise, the genetic background of the mice in the KLF2+/KLF4+/ crosses used in the current study was not well-defined, and this may contribute to the incomplete penetrance and variable expressivity of the KLF2-/KLF4-/ and KLF2-/KLF4+/- phenotypes.

KLF4 is expressed in endothelial and mesenchymal cells. The theory of genetic compensation by KLF4 in the absence of KLF2 is under consideration in research involving the adult vasculature. A study has shown that, like KLF2, KLF4 is induced by shear stress and activated by the MEK5/MEF2 signaling pathway. Using genomewide transcriptional profiling of HUVEC cells overexpressing KLF2, KLF4 or constitutively active MEK5, they showed that about ~60% of the genes activated by MEK5 were also regulated by either KLF2 or KLF4. This study suggests that there is mechanistic and functional conservation between KLF2 and KLF4 in the vascular endothelial cells (Villarreal Jr. et al., 2010). The complementary roles of KLF2 and KLF4 in vascular development also suggest KLF4 might attempt to compensate for loss of KLF2 in embryonic heart. It would be interesting to examine the amount of KLF4 mRNA expression in KLF2-/ embryos. KLF4 expression may be increased.

As described by previous studies in the lab, none of the three KLF2-/KLF4-/ embryos were alive by E10.5 as indicated by the absence of a heartbeat. Thus these embryos die before E10.5, suggesting a more severe phenotype than KLF2-/ alone. Dorsal aortae are not visible in cross-sections at E9.5, and hence their endothelial integrity could not be judged in KLF2-/KLF4-/- embryos. The E9.5 KLF2-/KLF4-/- embryos did not show any defects in the heart. However, this is not surprising because these embryos are in undefined mixed genetic background with approximately 50% FVB/N, rather than in a 100% FVB/N background.
CD31 staining of E9.5 KLF2/KLF4 DKO whole-mounts showed apparently normal capillary development as observed by previous studies in the lab (Benjamin Curtis, unpublished data). While E10.5 KLF2-/-KLF4-/-, E10.5 KLF2-/-KLF4+/-, E9.5 KLF2-/-KLF4-/-, and E9.5 KLF2-/-KLF4+/- embryos exhibited a primary head vein phenotype of variable morphological severity, the other vessels showed no obvious signs of hemorrhaging. In fact, the localized hemorrhaging along the primary head vein was the only obvious morphological defect that was observed. We currently have no explanation for the specificity of the vessels affected at E9.5, but a more severe and less specific hemorrhaging certainly occurs by E10.5.

In adult mice, blood vessels are supported in part by vascular smooth muscle that provides strength and contractility. In embryos, the primitive vascular network is supported by mesenchyme, the undifferentiated connective tissue that will ultimately differentiate into vascular smooth muscle cells during development (Reviewed by (Nehls & Drenckhahn, 1993)). Difference in mesenchymal cell density was not observed in E9.5 double knockout embryos along the primary head vein, where the hemorrhaging occurred. Glycosaminoglycans form an integral part of the extracellular matrix (ECM), contributing to the ground substance which cushions and supports the embryo through an extra-cellular framework. It is possible that a reduction in GAGs or other ECM proteins along the PHV may cause the localized hemorrhaging. Alcian blue staining could be used to assess the extracellular matrix composition.

Neither our results nor previous studies have reported vascular defects in KLF4-/- mice in utero. KLF2-/-KLF4-/- mice indicate that KLF4 plays a role in vascular development and is complemented by KLF2. KLF2 and KLF4 regulate expression of eNOS, which is a regulator of vascular smooth muscle tone in the mouse embryos (Benjamin Curtis,
unpublished results). Involvement of eNOS also suggests another mechanism by which loss of KLF2 and KLF4 results in vascular disintegrity. Embryos lacking KLF2 and KLF4 might not be able to withstand increasing fluid shear stress. This however is less probable since shear stress would play an important role in all the developing blood vessels and should affect the endothelium throughout the embryo or at least at the major vessels. There is no evidence that the primary head vein is under higher fluid shear stress than other blood vessels during mouse development.

Blood flow regulates heart development and vice versa. Thus vascular defects during development could impact the developing heart. Defining the simultaneous roles of KLF2 and KLF4 in vascular development might shade a light on mechanism of some of the heart defects. Congenital vascular malformations are comparatively more rare than congenital heart defects, however this is predicted to be due to the fact that most of them either present themselves as heart defects or do not survive to birth (Pierpont et al., 2001).
CHAPTER 6: DISCUSSION AND FUTURE DIRECTIONS

Cardiac development is a complex process. It involves several morphological and molecular events that are precisely synchronized. Extensive research in the morphological and molecular fields has been performed over the last several years, and a number of genes and signaling pathways that control the cardiac development program have been identified. In spite of this, the molecular etiologies of most congenital heart defects, including valve defects, are unknown. Our study confirms a position for KLF2 in the list of genes important for cardiac development.

Genetic background affects KLF2-/- cardiac phenotype

The importance of genetic background in cardiac development has been demonstrated in a number of studies. Sakata et al. studied Hey2 deficient mice and observed a spectrum of cardiovascular anomalies that varied in the BALB/c and C57BL/6 genetic backgrounds (Sakata et al., 2006). The previous studies indicated that KLF2-/- embryos die between E12.5 to E14.5. The KLF2-/- hearts showed myocardial thinning; however no other structural abnormalities were observed in those studies. Our study shows earlier and more severe effect of the loss of KLF2 gene on mouse heart development and viability. Thus the role of KLF2 in the morphology and function of the developing heart is also genetic background specific.

To confirm this, we back-crossed KLF2+/- animals onto FVB/N, C57BL/6 and mixed genetic backgrounds and looked at E10.5 embryos to compare the morphology and gene expression patterns. Table 6-1 shows variation in the KLF2-/- phenotype in different genetic backgrounds. KLF2-/- embryos in an FVB/N genetic background show the most severe
cardiac abnormalities of the 3 groups. C57BL/6 KLF2-/- embryos show all of the structural abnormalities that are seen in FVB/N KLF2-/- embryos; however the EMT defect is less severe. Surprisingly, in the mixed background, which is 50% FVB/N and 50% C57BL/6, none of the structural, functional or molecular abnormalities are evident, except for myocardial thinning. This indicates that strain-specific modifier genes differentially affect the downstream expression of Gata4, Tbx5, Sox9 and Ugdh and result in variable KLF2-/- cardiac phenotype. The modifier genes may be recessive because the majority of the cardiac abnormalities are observed in both the inbred strains but not in hybrid of the two strains. They may be different in FVB/N and C57BL/6 because both of the genetic backgrounds show cardiac abnormalities but they vary in severity and a mix of both of the backgrounds does not show most of the abnormalities reminiscent of genetic complementation.

An ambitious future study would be to find modifiers involved in embryonic heart EMT, using the FVB/N and C57BL/6 strains. FVB/N and C57BL/6 are inbred strains homozygous at all the alleles. FVB/N KLF2+/- adults would be crossed with C57BL/6 WT to obtain KLF2+/- animals in 50% FVB/N and 50% C57BL/6 background (F1) which will be further crossed with each other to obtain KLF2-/- in 50%FVB/N and 50% C57BL/6 background (F2). The animals obtained will have various combinations of FVB/N and C57BL/6 loci in the entire genome. The embryos obtained on mating the F2 50% FVB/N and 50% C57BL/6 KLF2+/- would be predicted to have a spectrum of cardiac defects. Embryos would be subjected to genotyping using a genome-wide panel of SNP markers, using the AV EMT defect as the binary trait (Rajagopal et al., 2007). There could be up to a few hundred candidate modifiers obtained from this step. However, the most important part would be narrowing down the list of the candidates by improving the resolution of SNPs in the linked region and re-genotyping. A literature review on candidates to see their cardiac development connection would also help shortlist them. Identification of modifier/s would significantly
enhance our understanding of the molecular basis of heart development and disease. A similar study has been done to identify modifiers in Fibronectin null mice in 129S and C57Bl/6 genetic backgrounds. Fibronectin null mice have inability of cardiac tube formation in the 129S background but not in C57Bl/6 (Astrof et al., 2007).

Table 6-1 Variation in KLF2-/- cardiac phenotype based on genetic background of the mice

<table>
<thead>
<tr>
<th>KLF2-/- phenotype</th>
<th>FVB/N</th>
<th>Mix</th>
<th>C57BL/6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absence of AV cushion EMT</td>
<td>++</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Atrial septal delay</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Myocardial thinning</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lack of normal cardiac jelly composition</td>
<td>+</td>
<td>-</td>
<td>N/A</td>
</tr>
<tr>
<td>Abnormal cardiac function</td>
<td>+</td>
<td>-</td>
<td>N/A</td>
</tr>
<tr>
<td>Cardiac genes dysregulation</td>
<td>+</td>
<td>-</td>
<td>N/A</td>
</tr>
</tbody>
</table>

+ indicates presence of an abnormality and – indicates its absence. ++ indicates more severe abnormality than +. N/A indicates that study was not performed.
The model (Fig. 6-1) summarizes the morphological and molecular contributions of KLF2 during cardiac development. We have shown that KLF2 binds to the promoter and regulates expression of Tbx5, Gata4 and Ugdh. These genes play an important role in various areas of cardiac development. KLF2 also regulates expression of Sox9; however no evidence was obtained in the ChiP assays to show its binding to the Sox9 promoter.

Gata4 null mice die between E8.5 and E10.5 (Kuo et al., 1997). E9.5 endothelial specific Gata4 knockout embryos show an accumulation of endocardial cells lining the AV canal, and AV endocardial cushions are hypocellular with respect to the mesenchymal cells. They show an absence of endocardial cell migration indicating abnormal EMT. This phenotype is similar to the KLF2 KO heart. This paper also shows that loss-of-function in certain components of the Erbb3- Ras- Erk pathway also results in the same AV endocardial cushion defects. Thus it is likely that KLF2 is upstream and regulates Gata4 and Erbb3 signaling pathway. The Erbb3 signaling pathway regulates EMT via regulation of the cardiac jelly synthesis (Rivera-Feliciano et al., 2006). At E9.5 the Sox9 KO also shows hypocellular endocardial cushions. However these embryos lack the endocardial cell accumulation evident in Gata4 and KLF2 knockout animals. The evidence suggests that Gata4 and Sox9 are downstream targets of KLF2 that are directly or indirectly instrumental in regulating AV endocardial cushion EMT.

Gata4 and Tbx5 heterozygous embryos have atrial septal defects (Maitra et al., 2004). At E10.5 KLF2-/- embryos show an absence of the atrial septum, unlike in the WT. This is too soon in the development to define it as an atrial septal defect. However it clearly indicates some delay or abnormality in atrial septation. Thus the KLF2 downstream targets, Gata4 and Tbx5, could be instrumental in regulating atrial septation. Tbx5-/- mouse embryos die around E10.5 – E11.5 (Basson et al., 1994). eNOS is one of the Tbx5 downstream target
genes in heart. Luciferase assays in endocardial progenitor cells show that Tbx5 enhances eNOS promoter activity (Nadeau et al., 2010). eNOS-/- embryos manifest septal defects like Tbx5-/- eNOS dysregulation and nitric oxide deficiency could be one mechanism by which mesenchymal cells apoptose around E10.5 and thus are unable to form the septum. However eNOS seems to be normally regulated in absence of KLF2. Thus there must be other cardiac gene(s) such as Smad that are downstream of Tbx5 and play an important role in atrial septation. Smads are regulated by Tbx5 and are important in developing cardiomyocytes (Kitamura et al., 2007).

Gata4 expression in mouse embryo begins at around E8.5 in the heart (Heikenheimo et al., 1994). It is shown to be expressed only in the extra-embryonic region prior to this developmental stage. Tbx5 is also expressed extra-embryonically until E8.0. Its expression in the embryo begins around E8.5 in the developing heart (Chapman et al., 1996). KLF2 expression in developing heart begins around E8.5 (Lee et al., 2006). The temporal expression pattern for all the three transcription factors is thus similar. Loss of KLF2, Gata4 (Rajagopal et al., 2007) or Tbx5 (Basson et al., 1994) in the mouse embryos results in cardiac defects at E9.5. This is thus consistent with the possibility that KLF2 regulates Tbx5 and Gata4.

KLF2-/- embryos also show a lack of cardiac jelly synthesis. Ugdh catalyzes the conversion of UDP-Glucose to UDP-Glucuronic acid (UDP-GA). UDP-GA is further converted to hyaluronic acid and other glycosaminoglycans by hyaluronan synthase 2 (Has2) (Walsh & Stainier, 2001). In humans, UGDH is known to be associated with valve defects (Hyde et al., 2012; Smith et al., 2009). Thus the downstream gene, Ugdh, seems to be a link between KLF2 and cardiac jelly synthesis.
Figure 6-2 represents a model describing a possible molecular mechanism by which KLF2 regulates cardiac development. The downstream targets of KLF2 identified in this work are not exhaustive. We expect there are other genes important in the cardiac development program that are regulated by KLF2. One of the future studies could thus be identification these genes. Microarray expression assays and subsequent confirmation by qRT-PCR, using mRNA from whole heart and/ or the AV region, will help in identifying novel KLF2 targets in the developing heart. A literature review could be used to tease out the relevant targets in cardiac structural and endocardial cushion development and cardiac jelly synthesis. ChIP and luciferase assays could be performed on these genes to see whether KLF2 directly regulates them. An elaborate signaling pathway regulated by KLF2 could be obtained from this study and would help us gain better insight in the molecular biology of congenital valve defects.

Further application based work would be to perform mutation/ polymorphism analysis by sequencing the KLF2 gene in patients with congenital valve defects and compare it to matched control individuals (Hyde et al., 2012; Smith et al., 2009). A large sample size would be required to make conclusive observations or increase the power of the study because KLF2 mutations are likely to be rare variants. The aim of this study would be to identify novel single nucleotide polymorphisms or other sequence changes altering KLF2 protein function. Once these mutations are identified, they can be further characterised using animal models. This piece of work would be an extremely important step, informative of the molecular aetiology, and could be used in drug and diagnostic development to prevent congenital heart and valve defects. Statins are shown to induce KLF2 expression in mouse and human endothelial cell lines. Thus they could potentially be used as a treatment in congenital valve defect patients with KLF2 mutations (Thienen et al., 2006).
Thus this work is important basic research identifying KLF2 as a potential regulator of cardiac development in humans.
Figure 6-1: Model representing the role of KLF2 in cardiac development through downstream targets Sox9, Gata4, Tbx5 and Ugdh in the FVB/N genetic background at E10.5. KLF2 binds Gata4, Tbx5 and Ugdh promoters, indicated by green structures. The dashed line from KLF2 to Sox9 indicates that Sox9 expression is regulated by KLF2. However no evidence of KLF2 binding Sox9 promoter was obtained. The downstream genes are instrumental in the various aspects of cardiac development indicated by images in the boxes.
Figure 6-2: Model representing the molecular mechanism regulated by KLF2 in cardiac development through downstream targets Sox9, Gata4, Tbx5 and Ugdh in the FVB/N genetic background at E10.5. KLF2 binds Gata4, Tbx5 and Ugdh promoters, indicated by green structures. The dashed line from KLF2 to Sox9 indicates that Sox9 expression is regulated by KLF2. However no evidence of KLF2 binding Sox9 promoter was obtained. The downstream genes further regulate genes and signaling pathways important in cardiac development. The question marks in red indicate that this is just a speculation and no studies have been performed by us or others to prove the same.


